University of Alberta

COMBINED OZONE-FUNGAL TREATMENT FOR DECOLORIZATION OF PULP MILL EFFLUENT

by

Elaine Joyce Wasylenchuk

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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Dr. D. W. Smith, supervisor

Dr. S. J. Stanley

Dr. J. J. Leonard

Date: 1 August 96

For my father,

Michael Wasylenchuk

ABSTRACT

Effluent from the aerated stabilization basin (ASB) of a bleached kraft pulp mill was treated with ozone followed by a biological system using the white-rot fungus *Phanerochaete chrysosporium* to determine if ozone enhanced biological colour removal in both batch and continuous flow systems. Basic conditions necessary for fungal decolorization were defined. Although one strain was able to reduce colour in ozonated wastewater without an additional carbon source, the other two strains tested required glucose for decolorization. When glucose was present, colour removal occurred at higher rates in raw ASB effluent than in ozonated samples. Iron added to the ozonated effluent strongly inhibited fungal colour removal. This metal leaching from the stainless steel rotating biological contactor (RBC) could be responsible for the lack of colour reduction in the bioreactor. Improved media, consisting of nutrients, buffer, and a surfactant, was investigated to determine which parameter enhanced fungal colour removal without an additional substrate such as glucose. Return of colour after ozonation was also investigated at varying temperatures and pH levels.

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LIST OF ABBREVIATIONS

ADMT	Air Dried Metric Tonne
Alpac	Alberta Pacific Forest Industries
AOX	Adsorbable Organic Halogens
AS	Activated Sludge
ASB	Aerated Stabilization Basin
ATCC	American Type Culture Collection
BCTMP	Bleached Chemi-Thermo-Mechanical Pulping
BKE	Bleached Kraft Effluent
BKP	Bleached Kraft Pulp
BKPM	Bleached Kraft Pulp Mill
BOD_5	5 day Biochemical Oxygen Demand
BOD_u	Ultimate Biochemical Oxygen Demand
CBQase	Cellobiose: quinone oxidoreductase
cfu	colony forming unit
ClO_2	Chlorine dioxide
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
CTMP	Chemi-Thermo-Mechanical Pulping
CU	Colour units
E_1	First alkaline extraction stage effluent
E_{op}	Alkaline extraction assisted by oxygen and peroxide bleaching
FSB	Facultative Stabilization Basin
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
HPC	Heterotrophic Plate Count
HRT	Hydraulic Retention Time
ICP	Ion Coupled Plasma
kg	kilogram
KHP	Potassium hydrogen phthalate
L	Liters
LC50	Lethal concentration for 50% of test fish
LiP	Lignin Peroxidase
M	Molar
m	meter

mg	milligrams
mL	milliliter
mM	millimolar
MnP	Manganese Peroxidase
$MnSO_4$	Manganese Sulphate
MyCoR	Mycelial Colour Removal
NaOH	Sodium Hydroxide
NF	Nanofiltration
NH_3	Ammonia
NH_4^+	Ammonium
O_3	Ozone
RBC	Rotating Biological Contactor
rpm	revolutions per minute
TC	Total Carbon
TCl	Total elemental Chlorine
TIC	Total Inorganic Carbon
TMP	Thermo-Mechanical Pulping
TOC	Total Organic Carbon
TON	Total Organic Nitrogen
TOX	Total Organic Halogens
TSS	Total Suspended Solids
UAMH	University of Alberta Mold Herbarium and culture collection
UF	Ultrafiltration
µm	micrometer, micron

1. INTRODUCTION

1.1 Background

Pulp mill effluent often contains high concentrations of colour causing compounds and chlorinated organics. Because these parameters are often difficult to remove by conventional sedimentation and biological methods, alternative methods must be developed to meet the increasingly stringent guidelines for pulp mill effluent.

Ozonation has been used in laboratory and pilot scale systems to reduce colour, chlorinated organics, chemical oxygen demand, and total organic carbon in pulp mill effluent. During ozonation, very little, if any, sludge is produced and no known harmful byproducts are generated, and the biodegradability of the waste is usually increased, making the process attractive to industry. However, the cost of ozone generation has limited the use of the process. As the efficiency of large scale ozone generation increases, the cost to implement an ozonation system has become less prohibitive.

Some type of biological treatment system is used in almost all Canadian pulp mills before discharge into a receiving water body. While aerated stabilization basins, activated sludge systems, and lagoons all have varying ability to reduce the BOD and total suspended solids of the wastewater before discharge, colour and chlorinated organics generally persist even after treatment. One type of biological system involving a white rot fungus has shown promise for use in pulp mill effluent treatment. This fungus, *Phanerochaete chrysosporium*, is able to degrade lignin to carbon dioxide, water, and a variety of metabolic products, given sufficient time. In wastewater treatment, most research has found that the fungi require an easily biodegradable carbon source in order to break down the high molecular weight tannin and lignin molecules mainly responsible for pulp mill effluent colour.

If ozonation breaks down the long chain molecules into simpler shorter compounds, the fungi may be able to use these fragments to degrade the remaining colour and chlorinated organics without requiring any additional carbon source. This would greatly enhance the desirability of the fungal treatment process as the additional expense of a carbon supplement would be eliminated.

Based on this hypothesis, a research project for Weyerhaeuser Canada Ltd., Grande Prairie Operations was designed using a combined ozonation and biological system for removal of colour and chlorinated organics from pulp mill effluent. Effluent wastewater

from the Aerated Stabilization Basins (ASB) at the Weyerhaeuser mill is typically high in colour (up to 1200 CU) mainly from the first alkaline extraction stage effluent (E_1) mixed with the other plant waste streams. Long chain molecules such as tannins and lignins which are predominantly responsible for the colour of pulp mill effluent do not significantly degrade in the ASB and thus are discharged to the Wapiti River near the plant.

Although the discharge of colour compounds may seem to be solely an aesthetic concern, colour can impact the ecological balance of an aquatic ecosystem and affect further use of the water by humans. Chlorinated organics, while not the adsorbable organic halides (AOX) such as toxic dioxins and furans that pulp mills were previously know to discharge, still may be associated with some toxicity when the longer chain molecules begin to slowly degrade into shorter chain, more bio-available compounds. Furthermore, some AOX compounds may be very slow to degrade and thus will persist in the aquatic environment. To meet future discharge guidelines for colour, Weyerhaeuser must incorporate new treatment processes, or improve existing ones to reduce the colour of their final effluent.

1.2 Objectives

The following objectives were proposed for the research:

1. Compare colour removal by three strains of *Phanerochaete chrysosporium* in raw and ozonated (ASB) treated pulp mill effluent with and without nutrient addition;
2. Reduce the variability present in the biological process;
2. Establish operating procedures for a new bench scale bioreactor;
4. Investigate fungal toxicity or decolorization inhibition by the presence of welded steel and trace metal contaminants;
5. Evaluate effect of extended aeration on colour, BOD, and pH of ozonated pulp mill effluent;
6. Evaluate fungal colour removal in an "improved medium" as described in earlier work (Mao 1996) and determine which factor(s) in the improved media composition have the greatest effect on decolorization; and
7. Investigate limitations to process for larger scale systems.

1.3 Alberta Environmental Protection discharge standards

Pulp mill discharge guidelines are established federally under the Pulp and Paper Effluent Regulations (Department of Fisheries and Oceans 1992) and under the Pulp and Paper Mill Effluent Chlorinated Dioxins and Furans Regulations (Department of the Environment 1992) then generally modified provincially to more stringent regulations. Table 1.1 compares federal guidelines under the Fisheries Act with parameters set provincially. Currently, only total suspended solids (TSS), biochemical oxygen demand (BOD), acute lethality, and dioxins/furans are regulated at a federal level. Since 1992, chlorine bleaching pulp and paper mills have not been permitted to discharge measurable quantities of 2,3,7,8-TCDD (dioxin) or 2,3,7,8-TCDF (furan) into the environment under the Pulp and Paper Mill Effluent Chlorinated Dioxins and Furans Regulations of 1992. Organochlorines such as AOX are not regulated federally because a scientific basis could not be established for setting a general level. As bioacccumulation, toxicity, and environmental persistence have not been directly linked with AOX, the regulation of this parameter may be difficult to justify in terms of environmental protection.

Alberta Environmental Protection determines discharge limits for each pulp mill based on the provincial water quality guidelines and on a mill specific assessment to evaluate the technology in place and the assimilation capacity of the particular receiving waters. Older mills in Alberta typically have less rigorous discharge standards than newer mills because of the advances in treatment technology that occurred. An operating license for a pulp mill not only details the compliance limits and schedule, but also includes testing procedures and monitoring requirements in the facility and the environment. Alberta Environmental Protection establishes limits based on loading (i.e. kg/day or kg/ADMt) rather than concentrations, thus preventing a mill from simply diluting its wastewater to the set standard. The water contaminant and parameter limits for the Weyerhaeuser effluent in Grande Prairie are shown in Table 1.2.

Monitoring requirements for the Grande Prairie mill are established by Alberta Environmental Protection. The monitoring schedule is shown in entirety in Appendix A. BOD_5, TSS, colour, and COD must be measured daily with composite samples. AOX is to be determined three times per week with a composite sample. Other parameters, such as temperature, flow, pH, and specific conductance are measured continuously with on-line monitoring.

Standards will continue to change, becoming even more rigorous, as technology improvements in treating the wastewater or in producing the pulp reduce the allowable concentrations of discharges. Environmental considerations and public pressures will also drive new standards, possibly even towards the absolute in minimizing environmental impacts: the effluent free or closed loop mill. As public opinion and choice of "environmentally friendly" products has already influenced how large corporations do business, pulp and paper mills have begun to see minimum impact technology not as a burden established by government, but as an effective marketing strategy that will win them larger profits.

Table 1.1. Comparison of federal and provincial government standards and reporting requirements for pulp mill effluent discharges. Adapted from Environment Canada information (Environment Canada 1996)

	Federal Government	Nova Scotia	New Brunswick	Quebec	Ontario
Control instruments	Effluent regs (Fisheries Act and CEPA)	No, Environmental protection Act-Waste permit system and federal lead	No, Clean Env. Act-Water Quality Regulation-certification of approval	Yes	Effluent regulations under Provincial EPA
Controlled parameters	TSS BOD Acute lethality Dioxins/furans AOX (not controlled)	Yes Yes Yes Yes No	Yes Yes Yes Yes No	Yes Yes Yes Yes Yes	Yes Yes Yes Yes Yes
Limits	TSS 11.25 kg/t [a] BOD 7.5 kg/t [a] Acute lethality LC_{50} ≤ 50% mortality in 100% effluent	Same as federal	Generally same as federal. Some certificates of approval tighter than federal.	8.0 kg/t 5.0 kg/t ≤ 50% mortality in 100% effluent	4.57 to 7.87 kg/t [c] 2.91 to 5.00 kg/t [c] ≤ 50% mortality in 100% effluent
CEPA	Dioxin Non-measureable ≤15 pg/L TCDD[d] Furan Non-measureable ≤50 pg/L TCDF[d] AOX - no standard			< 15 pg/L as TCDD[d] TEQ 1.0/2.0 [b] kg/t (1995) 0.8 kg/t (2000)	< 20 pg/L as TCDD[d] < 50 pg/L as TCDF[d] TEQ ≤ 60 pg/L 1.5 kg/t (1995) 0.8 kg/t (1999) eliminate (2002)
Section 7 of Federal Fisheries Act	7.(1)[a] Installing, monitoring, calibration	Yes	Yes	Yes	Yes
	[b] monitoring, reporting, monthly	Yes	Yes	Yes	Quarterly reporting due in 45 days
	[c] info on ownersip and changes	Yes	Yes	Yes	Yes

Table 1.1 (continued). Comparison of federal and provincial government standards and reporting requirements for pulp mill effluent discharges. Adapted from Environment Canada information (Environment Canada 1996).

	Federal	Manitoba	Saskatchewan	Alberta	British Columbia
Control instruments	Effluent regs (Fisheries Act and CEPA)	No, mills operate under Provincial EPA	No, permit system under Environmental Management & Protection Act renewable 2 yrs. Permit numbers only; still draft.	No, AB Clean Water Act. Operating permit/licences renewable every 3 or 5 years. New permits under Environmental Protection and Enhancement Act	Yes, Provincial regulations under Waste Management Act
Controlled parameters	TSS BOD Acute lethality Dioxins/furans AOX (not controlled)		Yes Yes Yes Yes Yes	Yes Yes Yes Yes Yes	Yes Yes Yes No Yes
Limits	TSS 11.25 kg/t [a] BOD 7.5 kg/t [a] Acute lethality LC_{50} ≤ 50% mortality in 100% effluent		11.2 kg/t 7.5 kg/t ≤ 50% mortality in 100% effluent	5.0 to 9.5 kg/t 3.0 to 5.5 kg/t ≤ 50% mortality in 100% effluent	5.0 to 18.75 kg/t daily 11.25 kg/t monthly 5.0 to 7.5 kg/t daily 7.5 kg/t monthly ≤ 50% mortality in 100% effluent
CEPA	Dioxin Non-measureable ≤15 pg/L TCDD[d] Furan Non-measureable ≤50 pg/L TCDF[d] AOX - no standard		≤15 pg/L TCDD[d] ≤50 pg/L TCDF[d] 1.5 kg/t annually	≤15 pg/L TCDD[d] ≤50 pg/L TCDF[d] 0.3 kg/t (New permit) 1.4 to 1.5 kg/t (Existing permit)	1.5 kg/t (1995) eliminate (2002)
Section 7 of Federal Fisheries Act	7.(1)[a] Installing, monitoring, calibration [b] monitoring, reporting, monthly		Yes Yes	Yes Yes	Yes for installing, no for maintaining and calibration Yes
	[c] info on ownersip and changes		Yes	Yes	Not stated

Abbreviations and notes to Table 1.1

EPA	Environmental Protection Act
CEPA	Canadian Environmental Protection Act
PPER	Pulp and Paper Effluent Regulations
AOX	Adsorbable Organic Halogens
TSS	Total Suspended Solids
BOD	Biochemical Oxygen Demand
LC_{50}	Concentration which causes 50% mortality of test organism
LC_{50} >100%	Survival of test organisms must be greater than 50% in 100% pulp mill effluent
TEQ	Toxic equivalents (TEQ=[2,3,7,8-TCDD] + 0.1 [2,3,7,8-TCDF])
RPR	Reference production rate

a	Factors used to calculate limits are in reality monthly max kg/t
	TSS = 7.5 * 1.5 = 11.25 kg/t monthly maximum
	BOD = 5.0 * 1.5 = 7.5 kg/t montly maximum
b	Softwood limits/Hardwood limits
c	Based on monthly average values
d	TCDD is 2,3,7,8-tetrachlorodibenzo-*p*--dioxin
	TCDF is 2,3,7,8-tetrachlorodibenzo-*p*--furan

Table 1.2. Alberta Environmental Protection water contaminant and parameter limits for the Weyerhaeuser effluent (Alberta Environmental Protection 1995)

Water Contaminants and Parameters	Schedule for Implementation	Water contaminant and parameter limits Mass in kg/day (unless otherwise specified)	
		Maximum Monthly Average Daily Discharge	Maximum Daily Discharge
BOD	Upon license issue	4510	8200
	January 1, 1994	4100	8200
	January 1, 1995	2460	4920
TSS	Upon license issue	4100	8200
AOX	Upon license issue	1230	2460
Colour	Upon license issue	131200	209 100
	January 1, 1994	114800	209 100
	July 1, 1997	73800	109 060
2,4-Dichlorophenol	Upon license issue	NA	231 g/d
Chromium	Upon license issue	NA	55
Sub lethal Chronic Toxicity (Ceriodaphnia dubia and Fathead minnow)	Upon license issue	greater than 6.25% NOEC at all times	
Acute Lethality (rainbow trout)	Upon license issue	more than 50% survival in 100% concentration test sample at all times	
Resins & Fatty Acids	Upon license issue	< 2 mg/L at all times	
Dioxin & Furan	Upon license issue	non-measurable at all times	
pH	Upon license issue	between 6.0 and 9.5 standard pH units at all times	

NA = Not Applicable

NOEC = No Observable Effects Concentration

TSS = Total Suspended Solids

BOD = Biochemical Oxygen Demand (5-day)

AOX = Adsorbable Organic Halogens

Colour: Assume 1 CU = 1 mg/L

1.4 Weyerhaeuser Canada, Grande Prairie operations

Built in 1972, the Grande Prairie, Alberta Bleached Kraft Pulp (BKP) mill has since undergone both a change in ownership and significant upgrades (Pryke *et al.* 1994). After assuming ownership from Proctor and Gamble, Weyerhaeuser Canada Ltd. now operates the 820 ADMt/d BKP mill using 100% soft wood, consisting of lodgepole pine, white spruce, and small quantities of balsam fir. The high grade pulp with uniform brightness (90% ISO brightness) is used in tissue and towel making, and in producing fine quality writing paper and some specialty products.

In 1990, Weyerhaeuser began to implement process modifications and improved treatment systems in response to forthcoming stricter environmental regulations. A 70% chlorine dioxide substitution in the first bleaching stage was added in 1990 then further upgraded to 100% substitution in 1992, thus completely eliminating the use of molecular chlorine during bleaching. A bleaching sequence of D(EOP)DED[a] is currently used in the mill. This conversion decreased the AOX in the final mill effluent to approximately 0.5 kg/ADMt, eliminated dioxins and furans from the effluent, and decreased the colour from 140 kg/ADMt at 70% chlorine dioxide substitution to about 80 kg/ADMt with 100% substitution. Foul condensate strippers installed in 1993 reduced the organic material, mainly methanol, discharged to the lagoons, lowering the influent BOD_5 from 34 kg/ADMt to 24 kg/ADMt as well as reducing the sulphide concentration by over 98%.

Effluent treatment at Weyerhaeuser's mill incorporates a settling pond to remove large fibers and other solids still in the liquid, leading into two aerated cells in series and finally a quiescent settling zone. Figure 1.1 shows a schematic of the ASB system with additions of chemicals marked. Weyerhaeuser has not made modifications for separate treatment of process streams, as many of the new mills use; instead it combines the various plant streams for biological secondary treatment in the ASB.

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[a] Bleaching sequence used refers to different stages or unit operations. D is reaction with chlorine dioxide in aqueous solution. EOP is caustic extraction with sodium hydroxide reinforced with elemental oxygen (O) and hydrogen peroxide (P). A conventional brightening sequence, DED, follows the first stage.

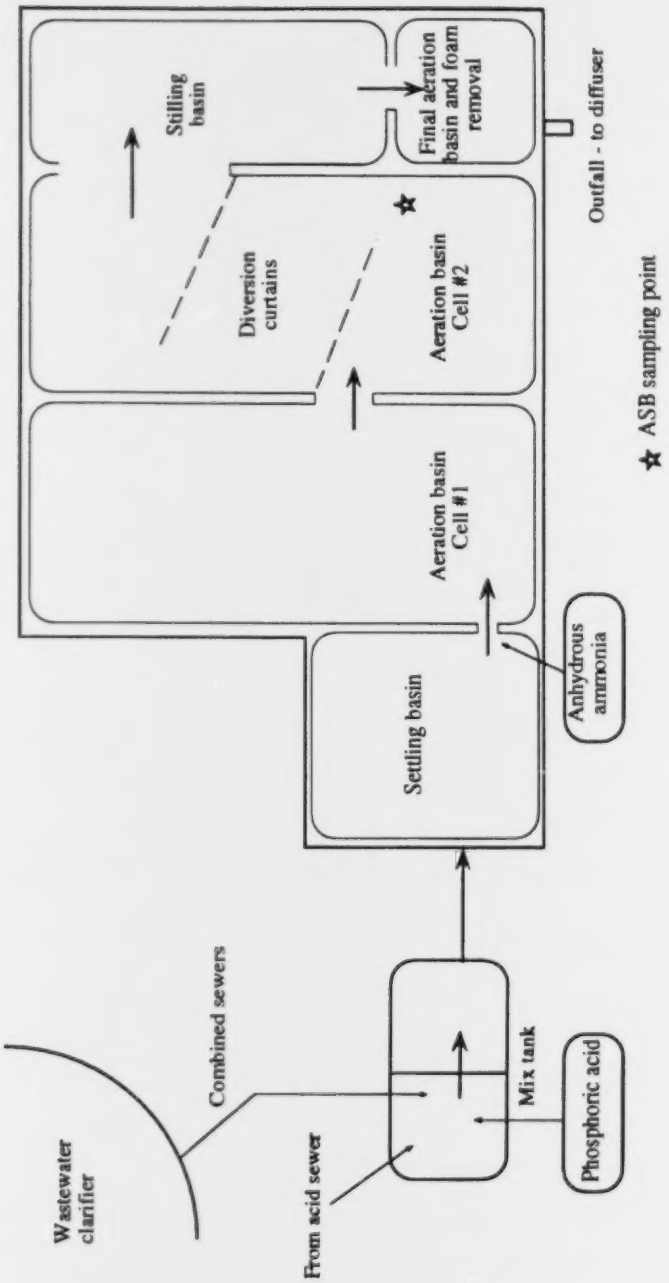


Figure 1.1. Aerated stabilization basins (ASB) and effluent treatment at Weyerhaeuser Ltd., Grande Prairie

The highly alkaline clarified effluent is mixed with effluent from the acid sewer to reduce the pH to levels that support biological treatment processes (7 to 8 pH units) then sent to the ASB. The ASB has eighteen 56 KW (75 hp) aerators in the first cell and nine in the second cell. To prevent short circuiting, polyethylene curtains were added to the second cell during the spring of 1993. Nitrogen and phosphorus are supplied to the ASB because the effluent is typically low in these nutrients. Even during the cold winter months in Grande Prairie, the ASB retains its heat, averaging between 30 to 35 °C mostly because the foam layer that the aerators create provides insulation. The average retention time for the effluent is 13 to 14 days at a flow rate of approximately 60 000 m^3/day (66 m^3/ADMt). Removal efficiencies across the ASB are shown in Table 1.3.

Table 1.3. Removal efficiencies of selected parameters from the influent to effluent of Weyerhaeuser's ASB treatment system

Parameter	Influent	Effluent	Removal Efficiency
BOD_5, mg/L	300 to 350	20	90 to 95%
TSS, mg/L	<20	15 to 20	--
AOX, mg/L	9 to 12	7 to 10	18%
Colour, CU	1000 to 1300	900 to 1200	<5%

The recent improvements made in plant processes and in the treatment processes have significantly improved the effluent quality. Although toxicity had been a problem in previous years (Lindsay 1993), the mill now consistently passes its acute and chronic toxicity tests.

Because the mill can discharge volumes that compose up to 10% of the Wapiti River flow, effluent quality is an important environmental and aesthetic factor. Numerous studies have been done to evaluate the health of the Wapiti River, the fish in the river, and other aquatic life (Alberta Environmental Protection 1987; Swanson *et al.* 1991; Weyerhaeuser Canada Ltd. 1993). Although a health warning still exists on eating certain fish species caught within the Wapiti River because of the residual levels of dioxins and furans still remaining in the flesh of the fish (The Edmonton Journal 1995), most studies concluded that the mill did not have a deleterious effect but that some habitats had still been altered.

By 1997, Weyerhaeuser must meet new compliance limits for BOD and colour, as shown in Table 1.4. Comparing the average performance over 13 months from January 1994 to January 1995, Weyerhaeuser can be seen to be well below their license limits in all cases for the 1994 standards. The stricter limit for colour discharge in 1997 is less than 20% above the average value for 1994. In order to consistently meet the license limits for colour, Weyerhaeuser must find a way to reduce the colour of their pulp mill effluent to account for possible daily variations in both raw water quality and treatment efficiency.

Table 1.4. Weyerhaeuser Canada Ltd. pulp mill effluent standards

		Average performance for Jan. 94 to Jan. 95	1994 Compliance standards for Weyerhaeuser	1997 Compliance standards for Weyerhaeuser
BOD	kg/d	1279	4100	2460
	kg/ADMt	1.5	5.0	3.0
TSS	kg/d	1010	4100	4100
	kg/ADMt	1.2	5.0	5.0
AOX	kg/d	485	1230	1230
	kg/ADMt	0.56	1.5	1.5
Colour	kg/d	63100	114800	73800
	kg/ADMt	73	140	90

Part of the pressure for increased effluent quality comes from newer mills in Alberta that use better technology in their plants and in their treatment processes to achieve low levels of pollutants. A comparison of Weyerhaeuser's effluent to other BKP mills in Alberta for the main categories of pollutants, AOX, TSS, BOD, and colour, is shown in Figures 1.2 to 1.5. Of the four mills shown, Weldwood and Weyerhaeuser are the oldest, beginning production in 1957 and 1973, respectively (Lindsay 1993). Both mills use exclusively soft woods. Daishowa-Marubeni and Alberta Pacific were constructed in the early 1990's and use either a combination of hard and soft woods or exclusively hard woods. All four BKP mills discharged better quality effluent in 1994 (based on monthly averages) than they were required to by their existing standards. Weyerhaeuser had the lowest TSS discharges, but ranked worst in terms of AOX and colour and second worst in BOD discharges (Weyerhaeuser Canada Ltd. 1995). While no changes to AOX discharge limits are set for any mill within the next two years, new 1997 limits for BOD and colour

have been established for Weyerhaeuser and for TSS and colour for Weldwood.

The environmental performance of Alberta BKP mills ranks in the top 20 to 40% of North American and Nordic mills (Mannisto *et al.* 1996). Mills included in the data base account for 44 million tonnes of BKP per year; Alberta mills account for only 3.4% of this production. Figures 1.6 to 1.9 show cumulative frequency curves for AOX, BOD, TSS, and effluent flow. Weyerhaeuser's average discharges, shown by the triangle on each graph, place the mill in the top 50%, 35%, 10%, and 25% for AOX, BOD, TSS, and effluent flow, respectively, on a per tonne basis. No comparison data was available for colour discharges.

Bleached kraft pulp mills are capital intensive and require significant expense to upgrade systems. Comparisons shown in Figures 1.6 to 1.9 include all BKP mills, regardless of age. Close to 90% of Canadian pulp and paper mills commenced production before 1971; these mills would not have had the same treatment technology available to them as newer mills now have. Weyerhaeuser's mill in Grande Prairie was constructed in the late 1960's and did not have the benefit of modern treatment advances. As upgrades are typically not as efficient as systems that are designed before the mill is built, older mills generally have higher pollutant discharges. In Alberta, even the earliest mills were built with secondary biological wastewater treatment which was beyond what past regulations required.

Although upgrades in Weyerhaeuser's Grande Prairie operations have improved the effluent quality, further measures are necessary to reduce discharges even more. Plant modifications and optimizations will not continue to yield as impressive changes as in the past; thus new alternatives are being investigated to meet the future effluent discharge requirements. Ozonation, membrane filtration, supercritical oxidation, biological treatment, and chemical precipitation are all options under evaluation for use at the Grande Prairie mill.

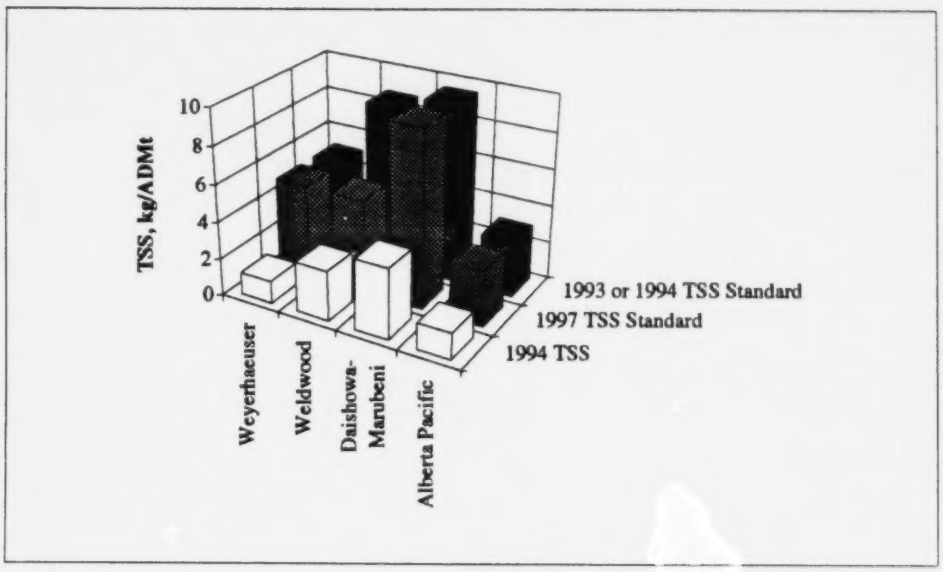


Figure 1.2. Comparison of current and new TSS standards and 1994 TSS discharges for Alberta Bleached Kraft mills

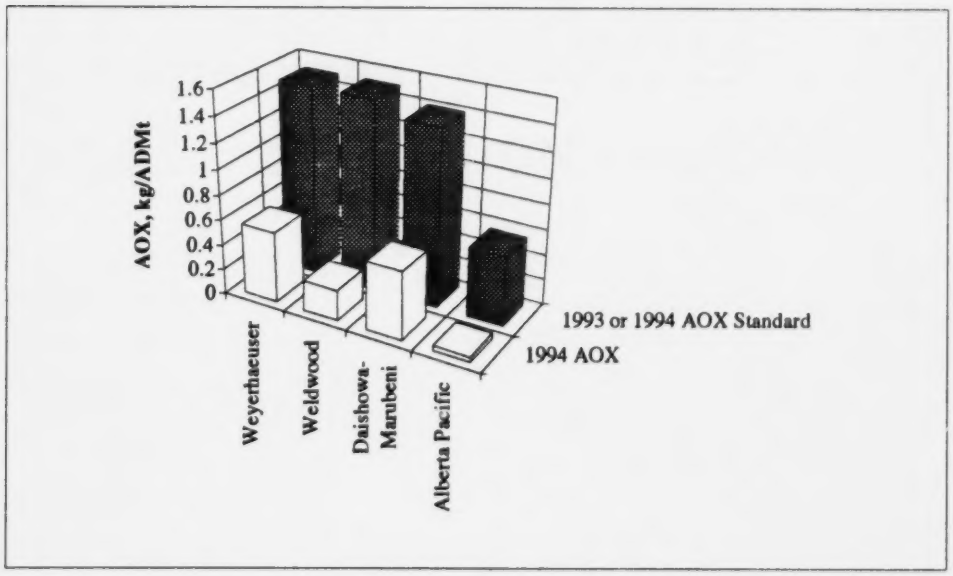


Figure 1.3. Comparison of current AOX standards and 1994 AOX discharges for Alberta Bleached Kraft mills

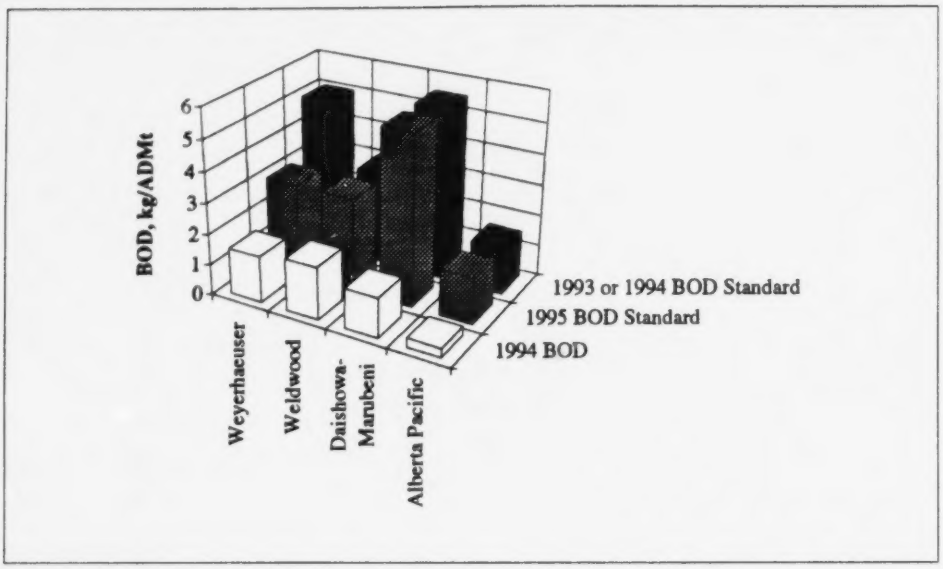


Figure 1.4. Comparison of current and new BOD standards and 1994 BOD discharges for Alberta Bleached Kraft mills

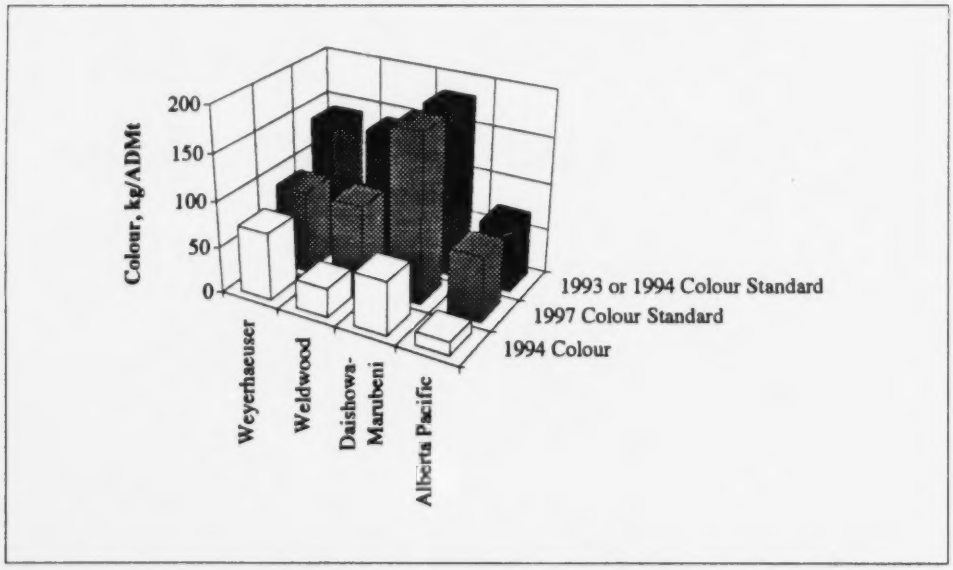


Figure 1.5. Comparison of current and new colour standards and 1994 colour discharges for Alberta Bleached Kraft mills

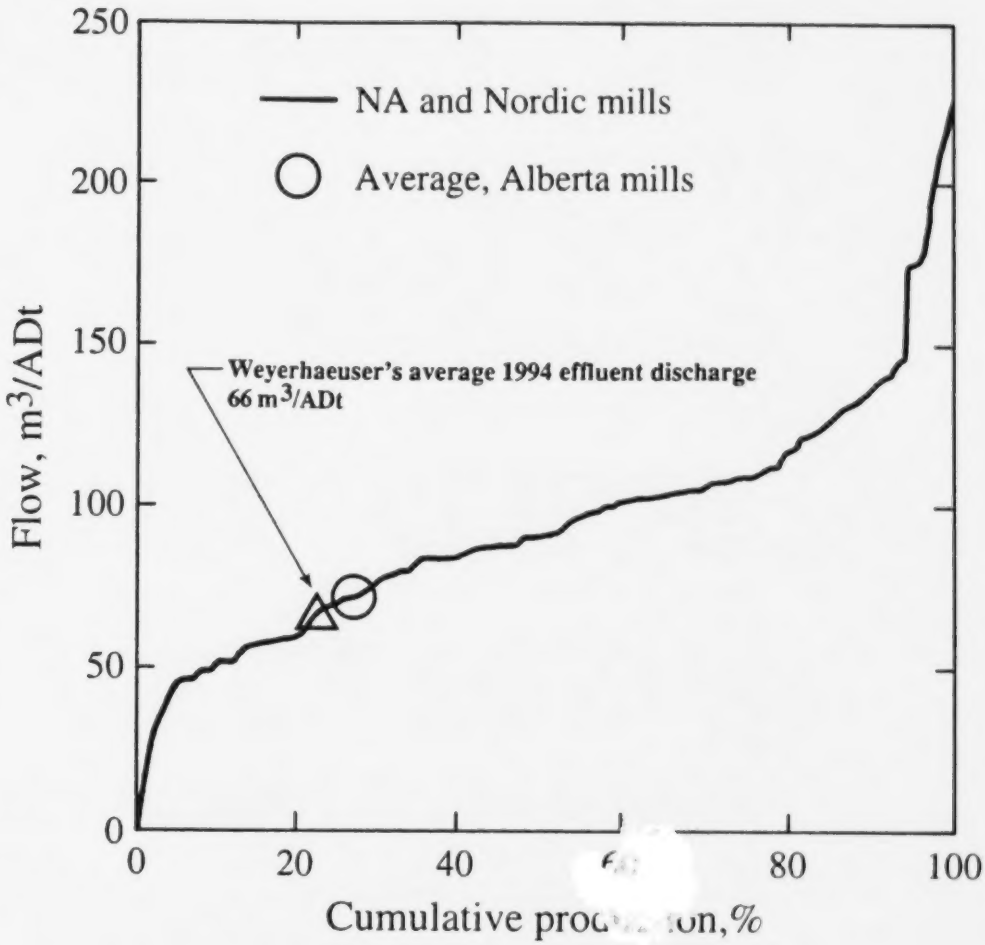


Figure 1.6. Effluent flows from North American and Nordic Bleached Kraft mills for 1994 compared with Alberta averages and Weyerhaeuser 1994 averages (adapted from EKONO/Duoplan Multi-Client Study 1995)

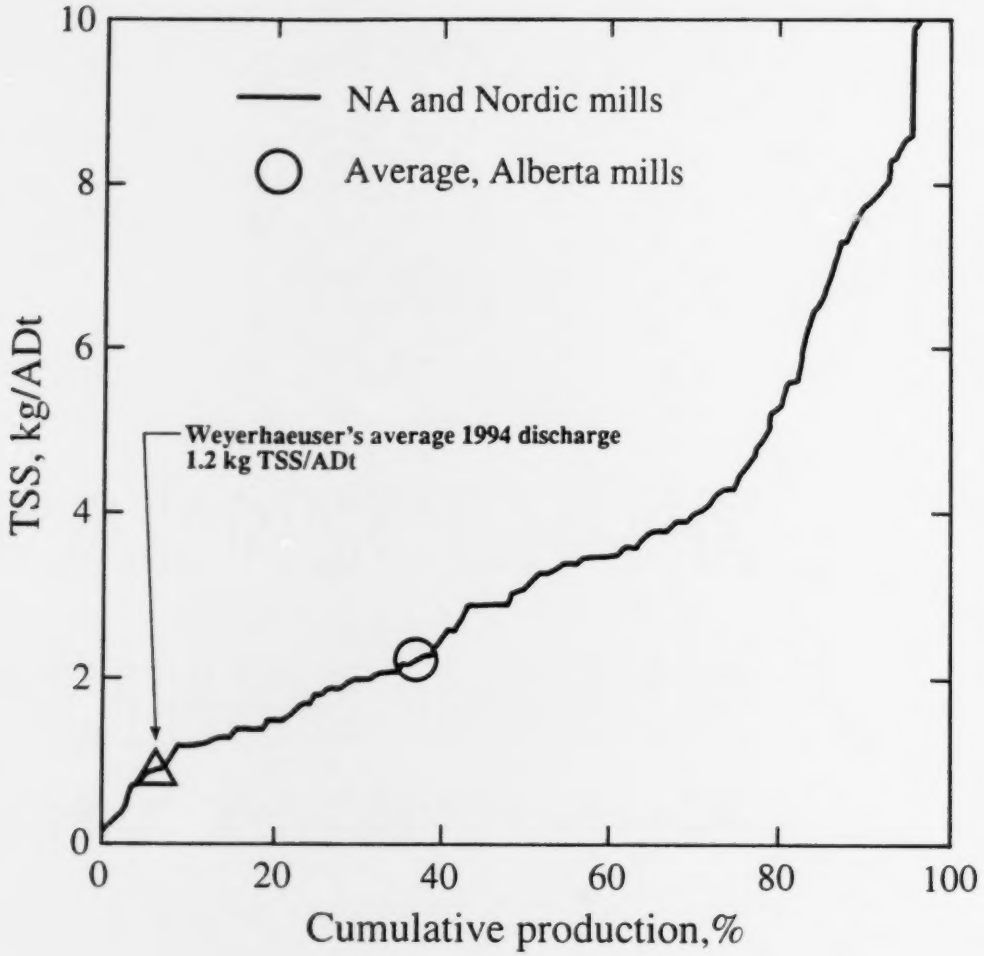


Figure 1.7. TSS discharges from North American and Nordic Bleached Kraft mills for 1994 compared with Alberta averages and Weyerhaeuser 1994 averages (adapted from EKONO/Duoplan Multi-Client Study 1995)

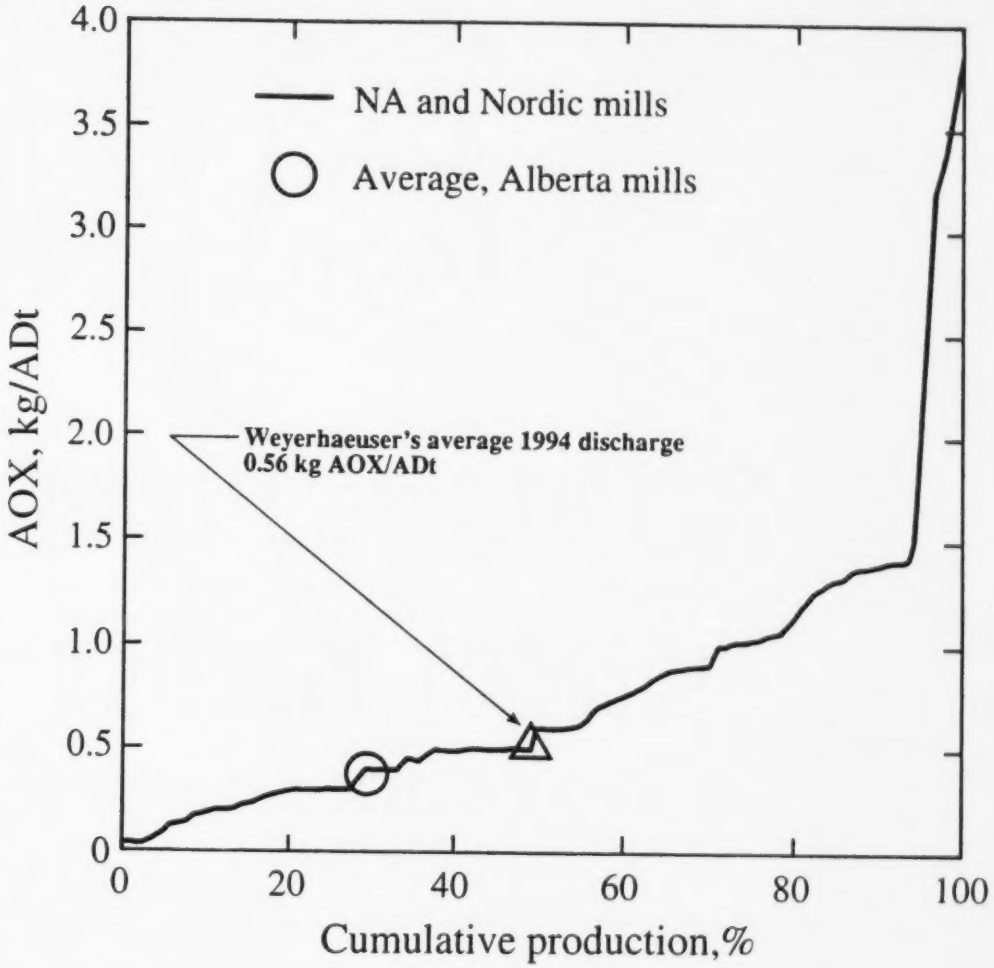


Figure 1.8. AOX discharges from North American and Nordic Bleached Kraft mills for 1994 compared with Alberta averages and Weyerhaeuser 1994 averages (adapted from EKONO/Duoplan Multi-Client Study 1995)

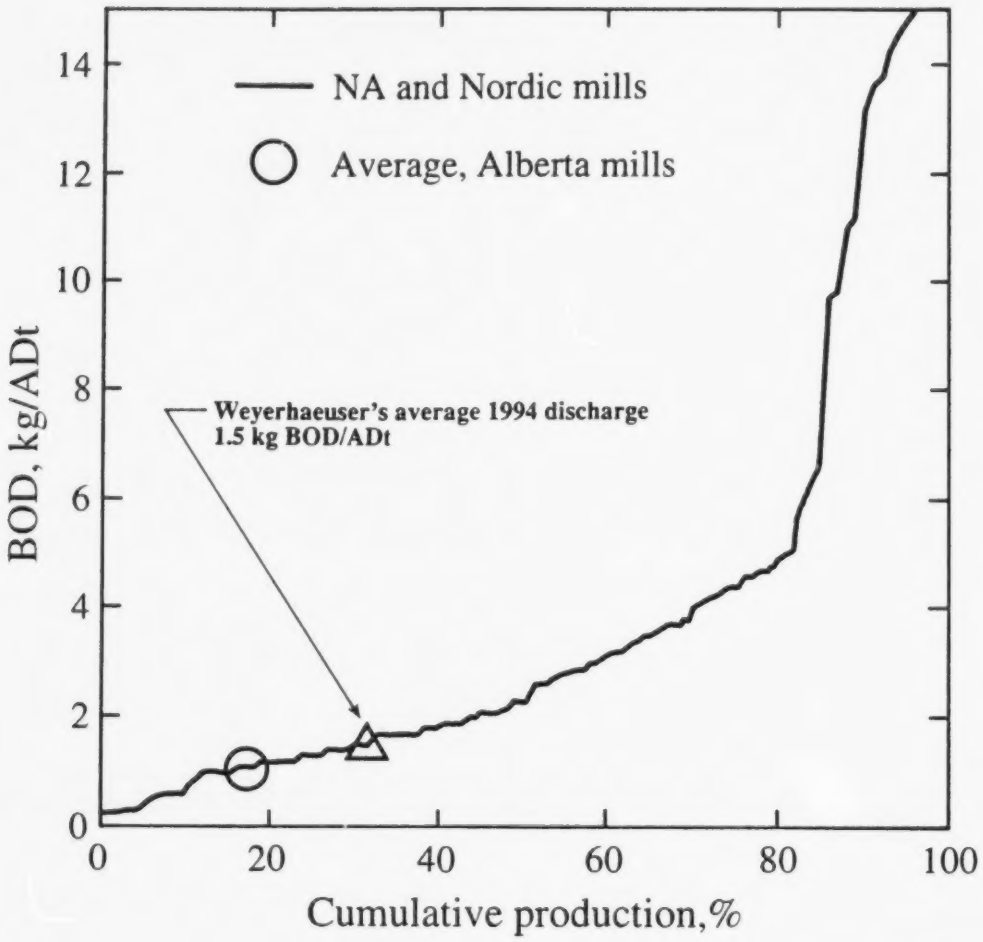


Figure 1.9. BOD discharges from North American and Nordic Bleached Kraft mills for 1994 compared with Alberta averages and Weyerhaeuser 1994 averages (adapted from EKONO/Duoplan Multi-Client Study 1995)

2. LITERATURE REVIEW

2.1 Characterization of waste streams

Two main methods of pulping are used in the pulp and paper industry. Bleached Kraft pulping (BKP) employs a white liquor mixture, sodium hydroxide and sodium sulphate, to cook the chips, producing black liquor as a product of lignin solubilization. This is burned to yield sodium carbonate and sodium sulphide smelt which is then reacted with quick lime to regenerate the original white liquor chemicals. Although the kraft process produces high strength fibers that are easily bleached, it typically has low yields and produces strong odours from the reduced sulphur compounds.

The second main method is mechanical pulping, which includes both thermomechanical pulping (TMP) and chemithermomechanical pulping (CTMP). Both involve pulping with mechanical energy and limited chemicals and heat. A high pulp yield is achieved, but the fibers produced are shorter and weaker than in the Kraft process and more difficult to bleach.

Principle water quality parameters of concern in pulp mill effluent are solids, oxygen demanding substances (both chemical and biological), toxicity, and colour. Each waste stream of a pulp mill will be responsible for different quantities and concentrations of pollutants. Table 2.1 outlines the various pulp and paper mill streams and their main pollutants.

The amount of colour and chlorinated compounds will depend on the processes used in the pulping and bleaching stages. Modified bleaching procedures, such as oxygen, ozone plus oxygen, ozone plus oxygen plus hydrogen peroxide, or chlorine dioxide bleaching can reduce or eliminate the formation of the chlorinated organic compounds.

Table 2.1. Source of pulp and paper making effluents and the main components present in each wastewater stream

Source of Effluent	Components present
Water used in wood handling and debarking	Suspended solids, BOD, colour
Digestor and evaporator condensates	Concentrated source of BOD and reduced sulphur compounds
White water from screening and cleaning	Large volume use containing BOD and suspended fibers
Bleach plant washer filtrates	BOD, colour, chlorinated lignins
Paper machine white water	Large volume use containing some BOD (usually recycled)

Pulp mill effluents can contain a variety of organic and inorganic materials that must be removed to a specified minimum discharge standard. Two principle components causing concern are colour and chlorinated organic compounds. The characteristic brown colour of many pulp mill effluents originates from the tannin and lignin compounds released during pulping. Colour is mainly an aesthetic consideration in the aquatic receiving environment but may have other effects, including reduced light transmittance for aquatic plants. Downstream industries and municipalities that treat the water for use may require increased amounts of coagulant and disinfectant to bring the coloured water up to appropriate standards. Chlorinated organics are often grouped under one measurement, adsorbable organic halogens (AOX). A number of toxic substances contribute to this group, such as chlorinated phenolics, dioxins, and furans. However, AOX is not a measure of overall toxicity because nonchlorinated compounds have been identified as responsible for sub-lethal toxic effects (McCubbin and Folke 1993). Resin acids, which occur naturally in softwood resins, are toxic to fish and frequently cause failure of effluent toxicity tests, even without a chlorine bleaching process being used.

The flow of effluent from pulp mills has decreased dramatically. In 1959 average water use from all types of pulp mills was 240 m^3/ADMt. It has since dropped down to 72 m^3/ADMt in 1988 for all types of mills and less than 40 m^3/ADMt for the most recently designed kraft pulp mills (Edde 1994). Table 2.2 shows the breakdown of flows from a typical modern softwood kraft pulp mill and the concentrations of COD, AOX, colour, and phosphorus in each stream.

Table 2.2. Modern softwood kraft pulp mill effluent characteristics (from Myréen 1994)

Source	Flow m^3/ADMt	COD kg/ADMt	AOX kg/ADMt	P g/ADMt	Colour %
Wood room	2	5	-	9	10
Fibre line	1	5	-	-	20
Bleach plant	30	25	1.0	36	40
Recovery system	2	5	-	-	-
Spills, etc.	5	8	-	-	30
From mill	40	48	1.0	45	100
From Treatment plant	40	22	0.5	20	100

ADMt = Air Dried metric ton of pulp

P = phosphorus

The bleach plant produces the largest amount of effluent with the highest contaminant loads, contributing large amounts of the plant's total discharge of colour, phosphorus, and COD. In addition, the bleach plant is the only source for AOX; alternative bleaching agents could eliminate or reduce the AOX from the plant. Developing a closed loop bleach plant would require consideration of water and chemical balances, corrosion, salt precipitation and removal of non-product substances (Myréen 1994). While internal collection and recycle may create benefits of water conservation and reduced discharge, these should be weighed against the increased capital and operating costs required to recycle the material. If the AOX compounds do not negatively impact the receiving environment, elimination of the bleach plant effluent for this reason alone may not be justifiable. The technology for effluent free pulp mills continues to develop and new pulp mills may face increasing regulatory pressures to close parts of their wastewater streams or achieve zero discharge with complete recycle.

2.2 Conventional pulp mill effluent treatment

Conventional treatment methods for pulp mill wastewater run the full selection of chemical, physical, and biological treatment methods. Any pollution control method in the pulp industry is usually very capital intensive and involves high operating costs. However, these costs are necessary to meet the governmental or self-imposed standards and are typically passed on to the consumer in the form of higher priced products. If the wastewater treatment method is well designed, the mill may see benefits from water

conservation, chemical recovery, and reduced energy consumption.

Primary treatment consists of solids separation through sedimentation, screening, or dissolved air flotation, with or without the addition of a coagulant chemical. Some reduction in BOD and toxicity is achieved through suspended solids removal. Secondary treatment is almost always biological and designed to reduce organic matter and nutrients in the wastewater. Tertiary treatment utilizes methods specific to problem compounds. Colour removal by lime coagulation, alum coagulation, ion exchange, activated carbon adsorption, or rapid infiltration would constitute tertiary treatment. AOX, if not sufficiently removed in the biological step, may also require tertiary treatment.

2.2.1 Biological Treatment

Secondary treatment typically uses biological processes such as aerated lagoons, activated sludge, anaerobic basins, biological filters, or rotating biological contactors (RBC) to convert waste material to less hazardous substances. Pulp mill waste does not supply enough easily biodegradable material and nutrients (nitrogen and phosphorus) to maintain an ideal microbial population for BOD removal. To ensure that the biological system is not nutrient limited, nitrogen and phosphorus are usually added to the system. Long retention time systems, such as aerated stabilization basins, require less nutrient addition than the high rate processes. In determining the nutrient addition requirements, the unused nutrient portion should be considered, as it could negatively impact the receiving environment.

Aerobic stabilization basins (ASB) use shallow lagoons and mechanical aeration to provide the oxygen for aerobic degradation of organic compounds and nutrients in the wastewater. The biomass is digested in the endogenous phase of the microorganisms, resulting in low generation of suspended solids. The ASB process is the most popular biological treatment system in North American pulp and paper mills, especially in Kraft mills (McCubbin and Folke 1993). The long detention times reduce the effects of variable wastewater characteristics and minimize maintenance; however, the extended times also make the system more susceptible to cold climates and temperature drops.

Settling of suspended solids containing AOX compounds, alkaline dehalogenation, and adsorption onto lime and bacterial solids were the principle mechanisms involved in achieving 65% AOX reduction in an ASB. Limited amounts of organic chlorine are

found in bottom sludges, indicating the AOX was mineralized and removed from the system (Stuthridge and McFarlane 1994). Resins and fatty acids collect in the foam layer that result from aerating, thus removing some toxicity from the water column. BOD reductions range from 40% in cold climates to greater than 90% in long retention well aerated lagoons (McDonald 1979; Luonsi *et al.* 1988).

Anaerobic systems, despite their limited use in Canadian mills, may have significant benefits over their aerobic counterparts. Lower space requirements, sludge production, and nutrient addition as well as biogas formation may increase the attractiveness of the anaerobic systems. Limited use, combined with the need for aerobic treatment to boost the oxygen levels before discharge may restrict the development of this technology in Canada.

Anaerobic processes have achieved higher AOX removals than aerobic treatment in bench scale tests using dilute BKP wastes (Ferguson 1994). With the aerobic system, 30 to 35% AOX removals were found; the anaerobic system achieved 40 to 45% reductions. Using an anaerobic/aerobic sequence, 50 to 55% of the AOX was removed and COD was reduced by 55 to 60% (Driessen and Wasenius 1994; Ferguson 1994). Several main mechanisms of AOX removal, including chemical or abiotic degradation after neutralization and biological processes were identified. The anaerobic biological removal was greater than the aerobic removal. Contrary to the study by Stuthridge and McFarlane (1994), sorption was identified as a relatively minor factor in both aerobic and anaerobic systems, possibly because of the differences in chemical addition between the studies.

Activated sludge (AS) is a high rate method that suspends the microbial mass in a mixing chamber, allowing the microbes to aerobically degrade the wastes. Short retention times are required for AS, but high floc carry over may increase suspended solids in the effluent if sufficient clarification is not provided. Disadvantages of the AS system include higher operating costs, sensitivity to shock loadings or pH changes, higher nutrient demands, sludge removal costs, and clarification requirements.

Facultative stabilization basins (FSB) and ASB systems outperformed AS in removal of total AOX compounds in laboratory scale tests, achieving 48%, 40%, and 22% removals, respectively (Hall and Randle 1992). When the fate of the chlorinated organics was assessed, biodegradation was identified as the most significant removal mechanism, accounting for 66 to 94% of all AOX removal. Less than 5% AOX was concentrated in

the sludges of any system and less than 0.5% loss was attributable to air stripping or volatilization. Longer retention times were key to improved AOX removal, thus accounting for the higher removal efficiencies by the ASB or FSB treatments (Hall and Randle 1994). Extended sludge retention time for AS system of 25 to 50 days may improve performance.

In an evaluation of four biological systems for treating combined effluent (Chen *et al.* 1974), the oxygen activated sludge and the rotating biological surface achieved 90% BOD reductions, while the aerated stabilization basin reduced BOD by 83% and the plastic medium trickling filter had the lowest BOD removals at 72%. Shock loadings and sudden pH changes were found to drastically reduce efficiency of all four of the processes. Colour and TSS were not affected by any of the treatments.

A new pulp mill in Alberta, Alberta-Pacific Forest Industries Inc. (Alpac), on the Athabasca River uses an activated sludge system for secondary treatment. The effluent quality is better than the other six mills in the province except in suspended solids discharge, where the mills average monthly TSS discharges are slightly higher than the lowest TSS discharger, Weyerhaeuser. Alpac's BOD, AOX, and colour emissions are well below the provincial standards set for the BKP mill. The system was designed to remove a minimum of 92% of the raw influent BOD_5 and 75% of the TSS using an extended aeration biological process (Alberta-Pacific Forest Industries Inc. 1989). Alpac's mills achieves better results than reported for Finnish AS systems which achieve 60 to 85% BOD_7 reductions (Luonsi *et al.* 1988).

2.2.2 Advanced Treatment Systems

Many paper mills have found advanced treatment systems necessary as more stringent regulations governing discharges developed. Tertiary treatment systems, although effective, add significant costs to the plant operations.

Precipitation of colour compounds, nutrients, and solids is a very efficient process, but it is also very costly (Möbius and Cordes-Tolle 1994). Colour removal can be accomplished with lime coagulation (Smook 1982) where the pH of the wastewater is raised to 11.3 or higher, thus precipitating organic colour compounds as calcium lignates. Activated carbon adsorption will also have high capital and operating costs, including regeneration or disposal of the material. Kallas and Munter (1994) compared ozone and

powdered activated carbon (PAC) removals of COD, AOX, and colour from three BKP mills with varying effluent characteristics. Ozone was found to be more selective in parameter reductions while PAC removed all compounds at approximately the same efficiency. However, on a cost basis, PAC was considered to be 10 to 20 times more expensive than ozone to achieve the same effluent quality.

Ion exchange can reduce bleach plant effluent colour, COD, and BOD by 100%, 95%, and 90% respectively for up to 15 bed volumes based on initial values of 14 000 ppm of Pt-colour, 2 400 ppm of COD, and 800 ppm of BOD (Anderson *et al.* 1974). A full size installation with 100 m^3/hour design flow rate was tested with results similar to the laboratory scale system. Slightly smaller total filtrate volumes exhausted the capacity of the bed (approximately 13 bed volumes).

Membrane filtration, both nanofiltration (NF) and ultrafiltration (UF), have gained popularity in research towards zero effluent mills. However, problems still exist with membrane life because of membrane fouling, scaling, tearing, and other failures (McCubbin 1992). Composite UF membranes may have longer lifetimes as they are water cleanable. Cho and Ekengren (1993) used modified commercial UF membranes to remove 94% of the AOX and 84% of the COD in alkaline extraction stage bleaching effluent. A membrane biological contactor removed 99.6% of the resin and fatty acids, 81% of the COD, and 74% of the dissolved organic carbon from simulated closed-mill mechanical newsprint whitewater (Tardif and Hall 1996). Total dissolved solids were less efficiently removed at 25%.

2.3 Treatment of pulp mill effluent with ozone

Ozone has been widely used in drinking water treatment as well as wastewater disinfection; in the pulp and paper industry, ozone was first considered as a bleaching agent for pulps and then as a treatment method for pulp mill effluents. Ozone has a strong oxidation potential which makes it ideal for rapid removal of compounds, such as chlorolignins, that are not easily oxidized by other methods. Although costs for ozone generation have previously prohibited large scale, high consumption uses, new, more efficient generators have made the process more attractive by reducing the costs from over 22 kWh/kg to 10 kWh/kg (Roy-Arcand and Archibald 1995).

While ozone use in pulp mill effluent treatment can effectively reduce colour, odour, and

COD, it also has other added benefits, including the following (Mohammed and Smith 1992; Bauman and Lutz 1974):

1. ozone oxidizes colour compounds either completely to CO_2 or to smaller organic fragments and produces little if any sludge material and no known harmful byproducts;
2. oxygen level of the effluent is increased after ozonation to saturation or above saturation;
3. bacteria, odour, and foaming agents are reduced;
4. few plant modifications are needed; the ozonation system can be added at different stages depending on the treatment requirements; and
5. ozone systems may be compatible with further biological treatment because large molecules will be broken down into more easily biodegradable fragments.

However, ozonation does have disadvantages, including the following:

1. ozone off-gas must be carefully monitored and controlled because it is a pollutant and a human health hazard;
2. pulp mill wastewater has a very high ozone demand;
3. ozone systems still have high capital and operational costs. The generating system would have to be over-sized in anticipation of treating widely varying effluent qualities;
4. suspended solids or other material may consume ozone without yielding any improvements in effluent quality; and
5. increasing dissolved oxygen to supersaturation (30 to 40 mg/L) may affect aquatic life in a receiving stream. Although higher levels of oxygen would generally improve water quality, excess oxygen during low flow has uncertain effects and may be toxic to fish and aquatic life in the near vicinity of the outfall.

The results of ozonation of pulp mill effluent are highly dependent on the initial characteristics of the wastewater. Different plant streams (Eop, combined effluent, or biologically treated secondary effluent) and different pulping methods (kraft, thermomechanical, or chemithermomechanical) affect the contaminant removals of ozone and the overall efficiency. Individual plants using similar processes have enough variation in their effluent quality that ozonation results for one specific effluent are not necessarily broadly applicable.

Ozonation of pulp mill effluents began to be investigated in the early 1970's for removal

of colour. Bauman and Lutz (1974) investigated ozonation of secondary effluent from a kraft mill. With a range of ozone doses, colour was reduced by 60 to 70% at 30 to 40 mg/L, BOD_5 increased by 100%, and COD decreased by 10 to 15%. Although total bacterial counts remained high after ozonation, coliform microorganisms were selectively reduced by 60 to 80% at 30 mg/L and almost 100% at 40 mg/L. Tests were also conducted to determine the persistence of high dissolved oxygen levels in the ozone treated effluent. Agitation of course was key to oxygen dispersion, but under slow mixing conditions, the dissolved oxygen remained above saturation for greater than two hours. Ozone levels above 40 mg/L were considered "absurdly high" and did not yield significant benefits.

Ozone treatment of combined kraft mill effluent before biological treatment yielded similar results in a number of studies. Although toxicity reduction was partly attributed to concurrent foam separation (Ng *et al.* 1974), ozone was found to have a pronounced effect on acute toxicity reduction in combined, untreated kraft effluent (Roy-Arcand and Archibald 1995). While untreated C/D and Eop stage bleach plant effluents showed little toxicity changes, biologically combined effluents increased in low-level toxicity by the creation of oxalate, acetate, and other low molecular weight organic acids.

BOD of untreated kraft effluents decreases with increased ozone dose (Ng *et al.* 1974; Nebel *et al.* 1974), after an initial small increase at low ozone doses. Mohammed and Smith (1992) correlated BOD decrease with TOC reduction. The smaller, more reactive compounds contributing to BOD were completely oxidized and removed from the effluent as CO_2. Biodegradability of bleach plant, primary, and secondary effluent was improved in the same study. BOD increase in secondary effluent was similar to that found by Bauman and Lutz (1974), with a 65 to 100% BOD increase attributed to complex molecules remaining after biological treatment being broken down into smaller biodegradable fragments.

Colour reduction has been a major focus of pulp mill effluent ozonation studies. Removal of colour compounds again occurs to different extents depending on the effluent stream or source. Mohammed and Smith (1992) found that colour removal improved after biological treatment where ozone colour removal effectiveness increases from bleach<primary<secondary, possibly because ozone demand for competing molecules was reduced during biological treatment. In contrast, other research reported no difference between biologically and no-treated effluents (Roy-Arcand and Archibald

1995). The conflict may be a matter of measurement. In terms of percentage reduction, biotreatment doubled the colour removals when compared to the ozonated wastewater before biotreatment. Such differences were not found in the earlier study that concluded biotreatment improved colour destruction.

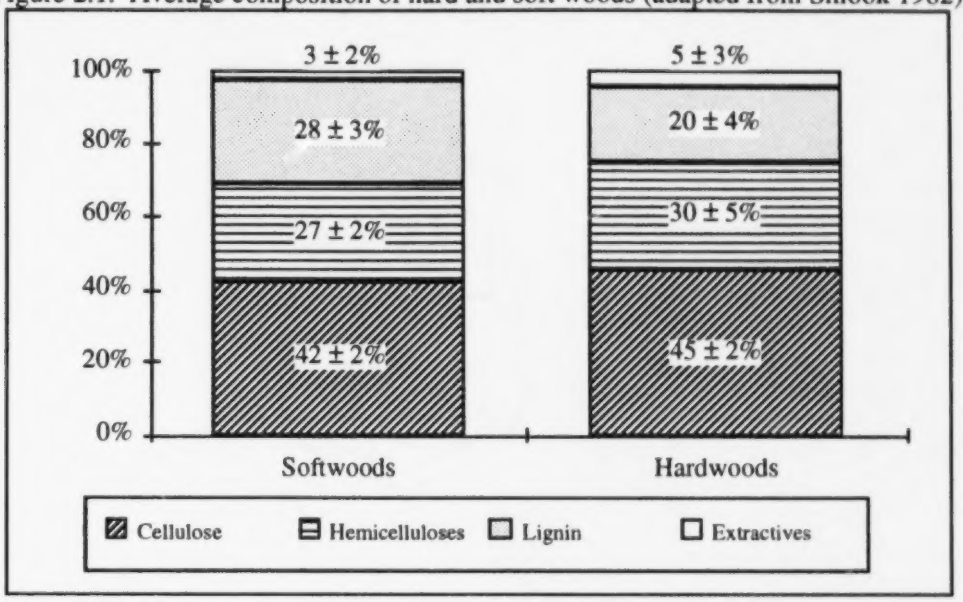
AOX reductions were positively correlated with initial concentration (Roy-Arcand and Archibald 1995). As biotreatment was found to improve AOX removals, the AOX fraction remaining after biotreatment was hypothesized to be more susceptible to ozonation. 100% removals of both colour and AOX would be virtually impossible to achieve with ozonation. Increasingly high ozone doses tend to remove smaller and smaller fractions of the contaminant. Low concentrations may also require high ozone doses for even marginal reductions, making the process expensive to achieve extremely high quality treated wastewater.

A study of combined ozonation-biotreatment of pulp bleaching effluents found that the degradation efficiency relative to the ozone consumed increased with the addition of a biological treatment stage (Heinzle *et al.* 1992). Over 70% AOX was removed by the combined system, improving the removals over both the individual biological and the separate ozone processes. Cyclic operation of the ozonation-biotreatment was suggested as the most beneficial operation mode to reduce ozone costs while still improving overall removals.

2.4 Lignin biochemistry

Wood is composed of three main polymers: cellulose, hemicellulose, and lignin, where the proportion of each varies among plant families and even among different strains of the same species. Hardwood and softwood trees contain different amounts of each component because their basic cell structures are distinct. Figure 2.1 shows a comparison of the average composition of softwoods and hardwoods.

Figure 2.1. Average composition of hard and soft woods (adapted from Smook 1982)



Lignin is a complex, three dimensional amorphous polymer with high molecular weight (1000 to 10 000) and with low aqueous solubility (Gottlieb and Pelczar 1951). Although the structure of hardwood and softwoods differ, both lignin types have repeating phenyl propanoid units joined by various carbon-carbon and ether bonds (Barr and Aust 1994). A representative drawing of part of a lignin polymer is shown in Figure 2.2.

More detailed descriptions of lignin chemistry and structure can be found in Pearl (1967), Sjöström (1981), Higuchi (1985), and Higuchi (1990).

To remove lignin from the cellulose fibers in kraft pulping, a series of bleaching and extraction stages are used. Soluble chlorinated lignin compounds are formed then extracted with NaOH and the process is repeated until the desired brightness of the pulp is attained (Smook 1982). Different bleaching agents and sequences will form different types of chlorinated organics. Free chlorine bleaching, used commonly until the last decade, produced dioxins and furans as byproducts of lignin chlorination. Chlorine dioxide tends to produce lower molecular weight organic chlorines, but the composition depends on both the extent of bleaching (the brightness or Kappa number reached) and the type of wood used. Gergov *et al.* (1988) found that the AOX concentrations in bleach plant effluents depend on the total elemental chlorine (TCl), which is partly determined by the extent of pulping and the use of other bleaching agents such as chlorine dioxide

that tend to reduce total AOX. Different species of woods were found to produce different amounts of AOX under the same pulping procedures. Hardwood pulping tended to create lower AOX concentrations than softwood pulp bleaching.

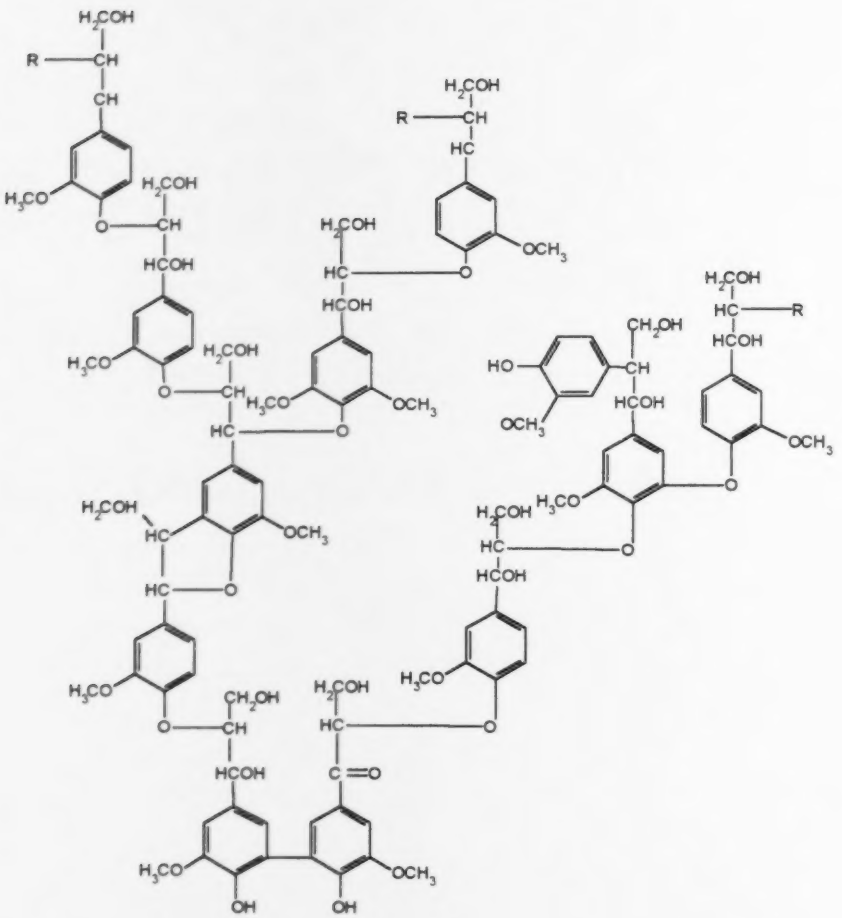


Figure 2.2. Main structures of a lignin polymer Adapted from Sjöström (1981) and Barr and Aust (1994). R denotes chemical structure extends beyond what is shown.

2.5 Use of fungi in colour and chlorinated organics removal

2.5.1 Screening of fungi for decolorization abilities

Many species of fungi have been tested for potential in decolorizing pulp mill effluent. Families or species of fungi are often chosen based on their natural habitat, the enzymes they produce, or previous related testing. Lignin degradation and colour removal from pulp mill effluent are obviously linked by virtue of chemical similitude. Research into both areas may be attempting to accomplish different end results, but the findings are usually significant to both areas. Screening of lignin degrading and decolorizing fungi are both relevant towards selection of a microbial agent for wastewater treatment.

Microbial degradation of wood is a natural process occurring in every forest environment. In a water medium, microorganisms, mainly bacteria and fungi, act to break down the lignin molecules into simpler molecules. Pulp mill waste contains an altered form of lignin which may be more resistant to microbe activity than normal lignin that has not gone through the pulping process (Kirk 1971). Fungi species have proven effective at lignin degradation (Gottlieb and Pelczar 1951; Liese 1970; Cooke 1970-71), typically outperforming bacterial populations present in waters. White rot fungi are a group of organisms that have developed nonspecific mechanisms to degrade complex mixtures, including lignin, completely to carbon dioxide, water, and other end products (Barr and Aust 1994). While white rot fungi may appear to be the ideal microbial agent for biological pulp mill treatment, difficulties may arise in the application. Enzymes are needed to catalyze the oxidation and reduction reactions in biodegradation; the lignin decomposition is a relatively slow process even with the catalysts present. The fungi usually lower the pH of their environment, which could make it less hospitable for other beneficial microorganisms. A final consideration would be maintaining the community of white rot fungi without overpopulation by competing microorganisms.

In screening 15 strains of white-rot fungi for decolorization ability of combined bleach plant effluent, Livernoche *et al.* (1983) tested decolorization on inoculated slants of effluent agar. The six fungi species showing colour removal were further tested in agitated liquid batch systems. *Coriolus versicolor* removed over 60% of the effluent colour (initial colour≈1000 CU) after six days growth in a 1% sucrose and mineral supplemented solution. While *Phanerochaete chrysosporium* removed 60% of the colour after four days, the colour increased with further incubation. However, the researchers conducted batch tests under agitation, which has been reported to be detrimental to colour removal by *P. chrysosporium* (Kirk *et al.* 1978). Non-shaken conditions may have

changed the selection of microorganisms for further study.

P. chrysosporium, under 100% oxygen aeration, and a *Rameria* species under air incubation, showed the highest decolorization rates and total removals after approximately 200 hours in another screening test (Galeno and Agosin 1990). *C. versicolor*, also tested, removed 80% of the original colour (14 500 CU) after 190 hours. Although neither *P. chrysosporium* nor the *Rameria* species was able to completely break down the high molecular weight chlorinated organics, both species significantly reduced the high molecular weight portion and created smaller organics in the process. Differences in the molecular weight distribution curves between the fungi tested may indicate the fungi use different metabolic processes in degrading the colour in E_1 effluent.

An extensive screening of 51 fungi strains was conducted in Brazil (Esposito *et al.* 1991) to investigate decolorization without an additional carbon source. When first alkaline extraction stage effluent, which contained 0.06% carbohydrates, was added to agitated cultures of fungal mycelium, 25 of the strains reduced colour by up to 73%, while the remaining 26 either had no effect or increased the final colour after five days. *Trametes* species, *Cordiopsis* species, *Coriolellus sepium*, *Xylaria* species, *Pycnoporus sanguineus*, *Cyathus pallidus*, *Cyathus striatus*, and *C. sitophila* all decolorized the effluent by 30% or greater. Glucose or some other simple carbohydrate is typically necessary for colour reduction (Kirk *et al.* 1976; Yin *et al.* 1989b; Archibald *et al.* 1990). This research suggested that formation of glucosidase enzymes enhances decolorization.

Studies of enzymes and their reactions have found that ß-glucosidase, also known as cellobiase, degrades cellulose through hydrolysis of the cellobiose molecules into two glucose units (Ghosh and Ghosh 1992; Wainwright 1992). Glucosidase has not been shown to affect larger molecules such as lignin derivatives. Although the enzyme may have been responsible for fungal utilization of carbohydrates (including cellulose) remaining in the effluent, the relationship between decolorization of effluent without an extra carbon source and production of an enzyme that exclusively degrades cellulose is unclear.

Lignin degradation, observed with a scanning electron microscope, was used to screen wood decaying white-rot fungi instead of decolorization tests (Blanchette 1984) and to examine relationships among fungal degradation of cellulose, lignin, and hemicellulose.

Coriolus versicolor was identified as a fungus that breaks down all cell wall materials. This factor may be important in biotechnology applications where preferential lignin degraders could be used for biopulping. Similar results were also found for *P. chrysosporium.*

Coriolus (Trametes) versicolor has been extensively studied as a white-rot basidiomycete with high potential for decolorization of pulp mill effluent. Archibald *et al.* (1990) found that kraft E_1-stage bleachery effluent could be decolorized by up to 80% after five to six days with a variety of carbon sources. In contrast to work done with *P. chrysosporium*, trace metals, nitrogen and carbon limitations, culture age, and veratryl alcohol did not affect rate or extent of decolorization by *C. versicolor* .

Twenty strains of white-rot fungi were tested for decolorization in batch studies with samples of combined bleach plant effluent from a sulphite pulp mill. The nine isolates of *T. versicolor* produced the highest decolorization (67 to 74% after six days) while several other fungi species also achieved up to 59% colour reduction. *T. versicolor* was further evaluated with a variety of co-substrates and under nitrogen limiting conditions. AOX was also reduced by 45% within two days by this strain of fungus.

Trichoderma species, while not belonging to the white-rot fungi group, may also be an effective colour removing microorganism. In continuous flow laboratory studies, at least 50% decolorization was maintained over six days using three different reactor types. In batch studies, *Trichoderma* was able to decolorize the effluent by 30% without an additional carbon source; after three days no further improvement was seen. Mycelial colour adsorption was not discussed as a possible explanation for decolorization.

Although wood decaying fungi are generally considered the only microorganisms that can completely degrade lignin and its derivatives, researchers have evaluated algae, other fungi families, and bacteria for potential use in biological treatment of lignin or pulp mill wastewaters. Table 2.3 outlines the microorganisms that are known to degrade lignin or decolorize wastewaters.

Bacterial genera capable of degrading lignin include *Flavobacterium, Micrococcus, Mycobacterium, Pseudomonas, and Xanthomonas* (Kirk 1971). Aerobic processes favour the destruction of long chain organics, although anaerobic systems have been effective over longer detention times. *Rhodococcus chlorophenolicus* is capable of degrading

many of the polychlorinated phenol compounds found in spent bleaching liquor of kraft pulp mills (Häggblom 1988).

Although white-rot fungi are the predominant wood decay organisms in a forest, the association among these basidiomycetes or ascomycetes, bacteria, and yeasts may be mutualistic, where bacteria and yeasts enhance wood degradation and in turn receive the nutrient by-products of wood decay (Blanchette and Shaw 1978). White-rot fungi may thus degrade lignin and cellulose faster and more efficiently in a heterogeneous microbiological system rather than isolating the microorganisms. This still does not eliminate possible competition for nutrients by other fungi, yeasts or bacteria in a mixed environment.

Table 2.3. Microorganisms known to degrade lignin or to decolorize pulp mill effluent

Microorganism	Reference	Substrate	Comments
Bacteria			
Bacillus cereus	Bourbonnais and Paice 1987	Dimethyl formamide-soluble portion of bleached kraft (E_1) effluent	Adsorption predominated in colour removal.
Flavobacterium	In Kirk 1971	Lignin	Extent of decomposition uncertain.
Micrococcus	In Kirk 1971	Lignin	Extent of decomposition uncertain.
Mycobacterium	In Kirk 1971	Lignin	Extent of decomposition uncertain.
Pseudomonas aeruginosa	Bourbonnais and Paice 1987	Dimethyl formamide-soluble portion of bleached kraft (E_1) effluent	Adsorption predominated in colour removal.
Pseudomonas ovalis	Kawakami 1975 in Bajpai and Bajpai 1994	Alkali lignin and kraft lignin sulphonate	Alkali lignin degraded better than kraft lignin sulphonate.
Xanthomonas	In Kirk 1971	Lignin	Extent of decomposition uncertain.
Fungi			
Aspergillus niger	Kannan and Oblisami 1990	Combined effluent samples from three mills, initial colour 1000 CU	56% decolorization after 2 days with 2% glucose concentration.
Bjerkandera adusta	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	12% colour removal after 6 days.

Table 2.3 (continued). Microorganisms known to degrade lignin or to decolorize pulp mill effluent

Microorganism	Reference	Substrate	Comments
Fungi, continued			
Coriolus (Trametes) versicolor	Bourbonnais and Paice 1987	Dimethyl formamide-soluble portion of E_1 effluent	16% of labeled organics converted to CO_2, 70% of colour removed.
	Royer *et al.* 1985	Kraft E_1-stage effluent, initial colour 5600 to 7000 CU	50% decolorization after 15 to 30 hours in a continuous reactor.
	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	90% colour and 45% AOX reduction in 3 days.
	Palleria *et al.* 1995	Kraft E_1-stage effluent	65% colour and 57% AOX reduction in 9 hours by immobilized fungus in fluidized bioreactor system.
	Roy-Arcand and Archibald 1991	Kraft E_1-stage effluent and ozonated (110 to 160 mg/L) kraft E_1-stage effluent	Ozone and fungus experiments run separately and in combination. Ozonation improved subsequent fungal treatment.
	Livernoche *et al.* 1983	Combined BKE and E_1-stage effluent, initial colour 1000 to 1200 CU	40% colour removal after 2 days, immobilized fungus removed 80% of the colour after 3 days. Various carbon co-substrates investigated.
	Galeno and Agosin 1990	E_1 effluent	80% colour removal after 190 hours.
Heterobasidion annosum	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	4% colour removal after 6 days.
Lentinus edodes	Esposito *et al.* 1991 Durán *et al.* 1994	BKP E_1-stage effluent Kraft E_1 effluent, 0.05% reductant sugars	73% colour removal after 5 days without any additional carbon sources; 13% mycelial adsorption of colour found. Pretreatment by photochemical means enhanced decolorization.
Merulius tremellosus	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	8% colour removal after 6 days.

Table 2.3 (continued). Microorganisms known to degrade lignin or to decolorize pulp mill effluent

Microorganism	Reference	Substrate	Comments
Fungi, continued			
Phanerochaete chrysosporium	Livernoche *et al.* 1983	Combined BPE, initial colour 1000 to 1200 CU	60% decolorization after 4 days in batch tests.
	Galeno and Agosin 1990	E_1 effluent	90% colour reduction after 190 hours and 100% oxygen. 70% reduction under air aeration.
	Esposito *et al.* 1991	BKP E_1-stage effluent	51% decolorization in 5 days without an additional carbon source.
Phlebia radiata	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	54% colour removal after 6 days.
Pleuotus ostreatus	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	59% colour removal after 6 days.
	Livernoche *et al.* 1983	Combined bleach plant effluent, initial colour 1000 to 1200 CU	40% decolorization after 8 days in batch tests.
Pleurotus sajor-caju	Bourbonnais and Paice 1987	Dimethyl formamide-soluble portion of bleached kraft (E_1) effluent	16% of labeled organics converted to CO_2, 70% of colour removed.
	Kolankaya *et al.* 1989	Bleach kraft plant effluent , initial colour 2450 CU	Colour removal dependent on pH, temperature, carbon source, hydraulic loading, disc rotation, oxygen tension, and nitrogen.
Polyporus versicolor	Livernoche *et al.* 1983	Combined bleach plant effluent, initial colour 1000 to 1200 CU	One strain (No. 171) removed 40% colour after 8 days in batch system. No. 97 had no effect.
Pycnoporus sanguineus	Esposito *et al.* 1991	BKP E_1-stage effluent	48% decolorization in 5 days without an additional carbon source.
Ramaria species	Galeno and Agosin 1990	E_1 effluent	90% colour reduction after 140 hours under air aeration
T. suaveolens	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	55% colour removal after 6 days.

Table 2.3 (continued). Microorganisms known to degrade lignin or to decolorize pulp mill effluent

Microorganism	Reference	Substrate	Comments
Fungi, continued			
Trichoderma	Prasad and Joyce 1991	E_1-stage effluent of hardwood pulp bleaching	85% color removals after 3 days cultivation with glucose. 68.6% colour removals without an additional carbon source. Continuous flow systems evaluated.
Xylaria hypoxylon	Bergbauer *et al.* 1991	Combined bleach plant effluent, initial colour 760 CU	45% colour removal after 6 days.
Algae			
Separate and mixed cultures of *Microcystis aeruginosa* and *Abaena flos-aqua* and a mixed algae culture from a pond in Fraser Valley B.C.	Lee *et al.* 1978	Biotreated BKME, approximately 150 to 250 APHA units	Up to 70% colour reduction in 2 months for acclimatized and unacclimatized algae. Mechanism not found to be adsorption.

2.5.2 White-rot fungi pollutant degradation

Biological methods have been used to treat wastes inadvertently through natural processes, and deliberately through engineered systems. Although bacteria are typically used in a wide variety of wastewater, solid waste, and hazardous waste treatment applications, the potential of fungi to biodegrade complex organics is becoming more well recognized and researched.

White-rot fungi are able to degrade a wide variety of xenobiotic compounds, ones with one or more aromatic ring and/or halogen substitution. These compounds are often toxic environmental pollutants that are persistent in many environments because few microorganisms exist that readily degrade the substances without succumbing to their toxicity. The ability of white-rot fungi to degrade lignin, a very complex polymer that differs greatly in composition, may create nonspecific mechanisms that also break down similarly structured pollutants. In addition, most fungi used extracellular enzymes that degrade compounds into simpler substances before they are taken into the cell itself. This collection of fungi can operate in conditions that many other organisms find intolerable because of the toxic compounds present. Chlorinated organics, especially substituted phenols, can be reductively dechlorinated by the white rot fungi.

Phanerochaete chrysosporium has been found to degrade many types of environmentally hazardous chemicals as shown in Table 2.4 (Lamar *et al.* 1992; Barr and Aust 1994). Although the researchers found that oxidative mechanisms are key to biodegradation, the fungus must have reductive mechanisms to break down already highly oxidized compounds. A route employing both veratryl alcohol and lignin peroxidase (LiP) was proposed for the breakdown of chlorinated organics such as carbon tetrachloride. In addition, a manganese peroxidase (MnP) enzyme may be responsible for the reduction reactions where a quinone reductase first may be necessary to convert the compounds into hydroquinones for the MnP mechanism.

Despite the role of white-rot fungi as the only eukaryote known to degrade fused ring aromatic hydrocarbons (Hammel 1989), research has not yet determined the full function of ligninolytic enzymes produced.

Table 2.4. Compounds degraded by *Phanerochaete chrysosporium*
Summarized from Lamar *et al.* (1992) and Barr and Aust (1994)

Polycyclic aromatic compounds
Benzo(a)pyrene
Pyrene
Anthracene
Chrysene
Phenanthrene
Thianthrene
Chlorinated aromatic compounds
Pentachlorophenol (PCP)
4-Chloroaniline
3,4-Dichloroaniline
Chloroaniline-lignin conjugates
2,4,5-Techlorophenoxyacetic acid
Polychlorinated biphenyls (PCB)
2,3,7,8-tetrachlorodibenzo(p) dioxin
2,4,5-Trichlorophenoxyacetic acid
Pesticides
1,1,1-trichloro-2,2-bis (4-chlorophenyl)ethane DDT
Lindane
Chlordane
Toxaphene
Aroclor 1254
Dyes
Crystal violet
Azure blue
Pararosaniline
Cresol red
Bromphenol blue
Ethyl violet
Malachite green
Brilliant green
Orange II
Tropaeolin O
Congo Red
Azure B
Munitions
TNT (2,4,6-trinitrotoluene)
RDX (Cylcotrimethylenetrinitroamine)
Others
Cyanides
Azide
Aminotriazole
Carbon tetrachloride

2.5.3 *Phanerochaete chrysosporium* morphological characteristics

Phanerochaete chrysosporium is a white-rot basidiomycete of the family Corticiaceae, order Aphyllophorales. The teleomorph name seldom used is *Sporotrichum pulverulentum*. The teleomorph and anamorph have both been isolated from fallen hardwood branches and from wood chip piles where it can cause significant losses. *Phanerochaete chrysosporium* is widely distributed in the world's temperate zone with specimens located in North America, Europe, and Iran (Burdsall 1985). *P. chrysosporium* falls into the white-rot fungi grouping and is capable of degrading both lignins and celluloses as well as modified derivatives of both. For use in the laboratory, and in treating pulp mill effluent, the asexual state of the fungus is used.

Other names that have been attributed to the same species of fungus include the following (Burdsall and Eslyn 1974; Stalpers 1984):

Chrysosporium pruinosum (Gilman and Abbott) 1962
Sporotrichum pruinosum (Gilman and Abbott) 1927
Chrysosporium lignorum (Berman and nilsson) 1966
Sporotrichum pulverulentum (Novobranova) 1972
Emmonsia ciferrina (Thirumalachal *et al.*) 1965
Emmonsia brasiliensis (Batista *et al.*) 1963
Sporotrichum dehradynense (Sarbhoy and Saksena) 1965

In 1974, Burdsall and Eslyn proposed that these names all represent the same species and should be named consistently as a new species of *Phanerochaete*. In applied work, the name of *P. chrysosporium* is used to avoid confusion. Stalpers (1984) found strains of *P. chrysosporium* were variable not only in morphology, but also in their ability to degrade lignin and in their production of phenoloxidase enzymes.

Growth patterns of *P. chrysosporium* have been studied not only to improve its performance in engineered systems, but also to gain fundamental knowledge of the lignin degradation that is vital to the carbon cycle of the environment. Ulmer *et al.* (1983) characterized the growth behavior of this fungus in chemostat and batch cultivation. Like many other types of fungi, *P. chrysosporium* displayed growth-rest cycles and recycling of nitrogen in nitrogen limiting conditions. During primary growth in batch reactors, the fungus increased its mycelial weight and consumed all available free nitrogen. In secondary growth, no increase in dry weight was seen, although carbohydrates continued

to be consumed. When cells established new growth every 12-15 days, increasing in weight for a few days before resuming static (no change) growth, the fungus may have been using nitrogen stored in older cells for new growth.

Secondary metabolism describes the growth phase after primary metabolism when all the requirements of cell growth have been fulfilled (Kendrick 1992). Lignin degradation only occurs after fungi have used up available nutrient sources and one or more factors become limiting. Secondary metabolites are not considered part of the ordinary existence of the microorganism as they have no role in growth or reproduction of the organism. They tend to accumulate because the fungus continues to produce them but cannot degrade them. Penicillin, cyclosporine, aflotoxin, and psilocybin are all secondary metabolites that have had or still have direct effects on humans, but also prove beneficial to the fungi too. *P. chrysosporium* produces enzymes in its secondary metabolism that are capable of degrading lignin, thus obtaining more nutrients from an otherwise unavailable source. Lignin degradation may only be a side route the fungi take to get at the cellulose and nitrogen stored in the wood (Kendrick 1992).

2.5.4 Decolorization and dechlorination abilities of *Phanerochaete chrysosporium*

Fungal decolorization of pulp mill effluent arose from research on lignin degradation because the colour causing compounds were known to be lignin derivatives from the bleaching process. While *P. chrysosporium* had been extensively studied for its ability to degrade lignin relatively rapidly, compared to other microorganisms, and for its active ligninolytic enzyme system, research soon began to focus on the fungus for possible development of an inexpensive effective colour removal process. When decolorization was found possible, simultaneous dechlorination of the high molecular weight chlorinated organics was considered as an added benefit of the proposed process.

Table 2.5 outlines research on decolorization of pulp mill effluents by *P. chrysosporium*. The strains of fungus used are listed in the table, but have not been cross referenced except when given by the authors. Therefore some research may have used the same fungus strains, but referred to them by different culture collection identification systems. Cross references are further discussed in the Materials and Methods section.

Table 2.5. Decolorization and dechlorination studies involving *Phanerochaete chrysosporium*

Reference	Strain used	Effluent sample	Parameters measured or varied	System	Results
Eaton et al. 1980	Burds ME-446	Initial colour ≈ 3500 CU, 4 BKP effluents tested	Colour	Agitated batch tests in 125 mL flasks	Agitation allows decolorization under lower oxygen concentrations. >80% decolorization after 10 days. Sodium ions did not affect colour removal.
Sundman et al. 1981	Burds HHB-6251 (ATCC 34540)	E_1 effluent concentrated to 30% volume and separated by ultrafiltration	Absorbance at 465 and 280 nm Molecular size distribution Ionization spectra	Stationary batch tests in 2.8 L flasks	Decolorization combination of chromophore destruction and polymer decomposition. Fungus shows no preference for molecular size in degradation process.
Campbell et al. 1982	Burds ME-446	Combined alkaline and acidic BKE, initial colour 1960 to 7600 CU	Colour Reactor type Detention time pH Disc velocity	Batch and continuous flow in MyCoR reactor	Batch MyCoR reduce 50 to 60% colour in 1 day and 75% in 2 days with initial colour 4000 CU. Continuous flow removes up to 75% colour with 3 day retention period.
Eaton et al. 1982	BKM F-1767 ME-446 HHB 6251	E_1 effluent from BKPM, initial colour 13 200 NCASI units	Colour BOD and COD Various sludge growth substrates	Stationary batch tests in 125 mL flasks, Batch tests in MyCoR reactor	BOD and COD reduced by 40% with 60% colour reduction after 5 days, but high sludge discharges increase BOD and COD.
Huynh et al. 1985	Burds ME-446	E_1 effluent from BKPM	Low molecular weight organics before and after fungus treatment	MyCoR reactor	Methylation, oxidation, and reduction are main degradation methods. Fungus degrades most low molecular weight components with reduction in total chlorinated organics.
Eriksson and Kolar 1985	*Sporotrichum pulverulentum* ATCC 32629	Effluent from chlorine and alkaline extraction of ^{14}C-labeled chlorolignins bleaching in lab	$^{14}CO_2$ produced Temperature varied	Agatitated batch cultures in 125 mL flasks	Fungus converted 40% of chlorine stage and 50% of E stage chlorolignins to CO_2. Higher rate of degradation was found at 28°C as compared to 38°C because of reduced oxygen solubility at the higher temperature.

Table 2.5 (continued). Decolorization and dechlorination studies involving *Phanerochaete chrysosporium*

Reference	Strain used	Effluent sample	Parameters measured or varied	System	Results
Vasudevan et al. 1986	Burds BKM F-1767	E_1 effluent from BKPM	Colour	Batch MyCoR reactor	Mineral solution added during decolorization phases improves colour removal. Tween 80 also improves colour removal by itself and in combination with nitrogen and mineral solution
Pellinen et al. 1988a	Burds BKM F-1767	E_1 effluent from BKPM, initial colour 8000 to 40000 CU	Colour Ammonium ion uptake COD Glucose concentration	Batch MyCoR reactor	Minimum glucose concentration required for decolorization is 2 g/L. Lignin-related COD decreased 32% and colour was reduced by 50 to 65% on average with one day HRT. Ammonium depleted very quickly.
Pellinen et al. 1988b	Burds BKM F-1767	E_1 effluent from BKPM, ultrafiltered and concentrated to 25% original volume	Total organic chlorine Glucose concentrations	Batch MyCoR reactor	50% decrease in total organic chlorine, 65% colour reduction, and 33% COD removals in one day. No low molecular weight degradation products found. Colour removal correlates with COD and TOCl removals.
Yin et al. 1989a	Burds BKM F-1767	E_1 effluent from BKPM, ultrafiltered and concentrated to different colour concentrations	Colour	Batch and continuous flow MyCoR reactor	Higher colour concentrations achieve better fungal colour removal rates, up to at least 23000 CU. Reaction kinetics found an initial rapid removal for 1 hour, then a zero order reaction rate, followed by a first order reaction. Overall colour removal was higher in continuous flow reactor, but batch performed better in the first 15 days.
Yin et al. 1989b	Burds ME-446	E_1 effluent from BKPM, initial colour 36000 CU	Colour COD Glucose added	Batch MyCoR reactor	Average colour removal >60% with glucose concentrations of 3 g/L or greater added. No change in removal with more glucose added. Critical glucose concentration was 1050 to 2100 mg/L. Only 1/3 of glucose was consumed in reactor.

Table 2.5 (continued). Decolorization and dechlorination studies involving *Phanerochaete chrysosporium*

Reference	Strain used	Effluent sample	Parameters measured or varied	System	Results
Prouty 1990	Burds BKM F-1767	E_1 effluent from BKPM, initial colour 8000 CU/L	Colour TSS Aeration rate	10 L aerated batch reactor	Maximum colour removal rate 1300 CU/L/day. Higher removal rates at initial colour concentration > 6000 CU/L. 4500 cm^3/min was optimal aeration rate.
Lankinen et al. 1991	Burds BKM F-1767 (ATCC 24725)	E_1 effluent from BKPM, hard and softwood effluents sterilized before use	Enzyme activities Colour removal AOX Various white rot fungi	Shake flasks under air atmosphere	Colour removal approximately 80% after 15 days for *P. chrysosporium*. Mycelium:effluent ratio and acclimatization are important to efficiency.
Michel et al 1991	Burds BKM F-1767 (ATCC 24725), ME-446 (ATCC 34541), mutants derived from ME-446	Synthetic E_1 effluent, initial colour 3000 CU	Enzyme production Growth patterns Glucose and nitrogen use	Shake flasks under oxygen atmosphere	Fungus mutant incapable of producing LiP or MnP did not decolorize effluent. High nitrogen blocked enzyme activity. MnPs play more important role than LiPs in decolorization of bleach plant effluent.
Fukui et al. 1992	Burds BKM F-1767	E_1 effluent produced in lab and E_p effluent from BKPM	Molecular weight Colour AOX Toxicity	Shake flasks flushed with oxygen every second day	Colour removal was most highest in the MW>10000 fraction. Molecular weight decrease after decolorization increased toxicity in high MW fraction, but decreased toxicity overall. AOX decreased in all fractions studied.
Berbauer and Eggert 1992	K-3	Combined effluents from sulphite mill (CEH) and (EOP) stages and bleachery effluent (HDEH)	Colour Absorbance at 280 nm Molecular size distribution	Stationary 1L flasks containing 250 mL liquid	Colour and absorbance at 280 nm reduced in CEH and HDEH effluent but not in EOP. Molecular weight distribution changed most in CEH and HDEH with only small changes in EOP.
Mao 1996	ATCC 24725	Combined ASB effluent from BKPM and ozonated ASB effluent from BKPM	Colour Molecular size distibution AOX BOD, TOC, COD	Stationary batch cultures and RBC system	Ozone pretreatment allowed fungal decolorization (>65%) and dechlorination (>50%) in an RBC system without an additional carbon source.

Varying decolorization efficiencies are mainly a product of the different test conditions, strains of fungus used, and cosubstrate supply. Results range from 80% colour removal in 10 days (Eaton *et al.* 1980) to greater than 65% in one day (Pellinen *et al.* 1988a and Pellinen *et al.* 1988b). On average, most systems were able to remove greater than 50% of the initial pulp mill effluent colour within four days.

Like lignin degradation, decolorization and dechlorination of pulp mill effluent requires an additional carbon source present and is enhanced by higher oxygen concentrations and optimum temperatures (Eaton *et al.* 1980). Nitrogen was also found to inhibit decolorization as in lignin containing cultures. Because the same conditions favour both processes, ligninolytic enzymes were deemed responsible for decolorization as well as lignin biodegradation. The mechanisms involved in fungal decolorization of pulp mill effluent were studied to determine if actual destruction of the colour bearing material was taking place or whether adsorption mechanisms dominated. Decolorization was not found to occur through adsorption because the mycelium remained light coloured and no colour could be extracted from the cell wall (Sundman *et al.* 1981). Chromophore destruction in the polymer and decomposition of the chromophore carrying polymer both occurred in decolorization by *P. chrysosporium*. The fungus showed no specificity for molecular weight during degradation. Fukui *et al.* (1992) also found that in E_1 effluent, all molecular weight fractions were degraded approximately the same. However, when the effluent was fractionated before fungal decolorization, the high molecular weight fraction showed the greatest colour reduction after two days of fungal treatment. Over 80% of the colour was emoved from this fraction compared with 65% in each of the medium and low molecular weight fractions. AOX removal showed a reverse trend. Although the whole effluent again showed fairly equal removals in all fractions, the highest AOX degradation occurred in the low molecular weight fraction (57%) and the least in the high molecular weight fraction (38%). In both cases, lower molecular weight degradation products were present in the treated samples.

During treatment with *P. chrysosporium*, the BOD and COD of the effluents were reduced when the cosubstrate was neglected from the parameter determination. With a 60% colour removal using primary sludge as a substrate, BOD and COD were both reduced by approximately 40% (Eaton *et al.* 1982). Similar results were found measuring only lignin related COD changes, which decreased 32% in two days (Pellinen *et al.* 1988a). However, at the concentration of glucose used as a cosubstrate, the final COD would be greater than 3600 mg/L. Decolorization, dechlorination, and COD decrease

were all found to be statistically correlated, suggesting a metabolic link among these processes, even though they have different reaction kinetics (Pellinen *et al.* 1988b). Detoxification of bleach plant effluent may also occur during decolorization when low molecular weight chlorophenols contribute to toxicity. Toxicity may be increased in some fractions of bleach plant effluent as high molecular weight recalcitrant compounds are degraded into more toxic low molecular weight chlorinated compounds although total toxicity decreases (Berbauer and Eggert 1992).

Initial colour of the pulp mill effluent impacts the rate of colour removal by *P. chrysosporium*, with higher rates with higher initial colour concentrations. Investigations of reaction kinetics in both batch and continuous reactors with highly coloured wastewaters (colour > 20000 CU) have shown an initial rapid removal stage followed by zero order reaction then a first order reaction (Yin *et al.* 1989a). Improved decolorization was also found when the initial colour was greater than 8000 CU in a batch reactor (Prouty 1990). However, at higher colour concentrations, the colour removal lifetime of the fungus may be reduced (Campbell *et al.* 1982 and Yin *et al.* 1989a), possibly because some nutrient becomes limiting, effluent toxicity accumulates, or slime layer development reduces oxygen and nutrient transfer. The majority of studies on decolorization of pulp mill effluent have used concentrated wastewater streams to maximize the removal rate. Combined effluent is lower in colour but may be more biodegradable by a fungal system because toxicity is typically reduced. Combined effluents, both before and after conventional biological treatment have been investigated for decolorization by *P. chrysosporium* (Mao 1996) with some success, although the continuous process was not run until failure.

Batch systems, as shown in Table 2.5, dominate in use for laboratory evaluation of decolorization and typically consist of small Erlenmeyer flasks flushed with oxygen periodically. A few bench-scale reactors have been developed and patented for assessment of more realistic conditions. The Mycelial Color Removal (MyCoR) process has been used extensively in both batch and continuous studies. This rotating biological contactor (RBC) consists of a Plexiglas 2.5 L reactor with eight scored plastic discs for fungal attachment. A clock motor rotates the discs, submerging them approximately 40% in the effluent. Aeration is provided by a steady flow of oxygen through the closed reactor (Eaton *et al.* 1982). Other reactor types have been investigated, including simulated aerated lagoon types (Campbell *et al.* 1982 and Prouty 1990), upflow columns, downflow columns (Campbell *et al.* 1982) and trickling filters . Different species of

fungi lend themselves better to alternate treatment schemes. *T. versicolor* has been immobilized in foam or gel beads and used in fluidized bioreactor systems (Livernoche *et al.* 1983; Pallerla *et al.* 1995; Pallerla and Chambers 1996). Considerations in selecting a reactor design include the following:

1) oxygen transfer,
2) decolorization rate achievable,
3) agitation,
4) effluent/fungus contact,
5) microbial contamination or spore release,
6) temperature control,
7) reaction kinetics and best suited flow regime,
8) biomass production and carryover,
9) hydraulic retention time,
10) short circuiting minimization, and
11) hydraulic loading rate.

Decolorization and dechlorination of pulp mill effluent with *P. chrysosporium* is possible given the optimum set of conditions for growth and decolorization, and given sufficient reaction time. The studies outlined here show the progress that has been made in this research area. Even after over 15 years of evaluation, this process has not been used either in a pilot or full scale plant. Problems of cost, adaptation to existing plant processes, efficiency, consistency, and high oxygen demand in the effluent must all be addressed before the fungal decolorization process can be applied to more than laboratory research.

2.5.5 Temperature and pH

Temperature regulates the reaction rates of most microorganisms. *Phanerochaete chrysosporium* has been shown to degrade lignin most efficiently and grow most rapidly at 37 to 40°C (Kirk *et al.* 1978). Some lower temperatures have been investigated to determine lignin degradation rates in marsh-like conditions (Leszkiewicz and Kinner 1985). Of the three temperatures tested, 15, 22 and 35°C, the highest temperature produced the maximum lignin degradation rate. The researchers also found that temperature had a stronger effect than oxygen concentrations.

Growth and decolorization may have different optimum ranges of pH and temperature

(Kirk *et al.* 1978; Eaton *et al.* 1980). Optimum growth occurred at pH 4.3 to 4.8 and 40°C, while decolorization could take place in pH 3.0 and at temperatures of 28°C, provided the optimum pH range was present during growth. Because the fungus produces acids during decolorization, thus lowering the pH, the effect of higher pH values on colour removal is uncertain and has not been evaluated. The difference between growth optimums and decolorization/lignin degradation optimums suggests a different stage of growth or metabolism occurs in each instance.

2.5.6 Dissolved oxygen requirements

Oxygen has been shown to be a necessity for an oxidative process such as fungal decolorization. Whether the *Phanerochaete chrysosporium* could be aerated with oxygen depends on if improved decolorization rates under higher oxygen concentrations outweigh the extra cost over air addition.

Kirk *et al.* (1978) first investigated effects of varying oxygen concentrations on lignin metabolism by *Phanerochaete chrysosporium*. While growth was similar under 5%, 21%, or 100% oxygen gas concentrations, lignin conversion to CO_2 occurred most quickly at 100%. At 5% oxygen, mycelial growth continued, but no lignin decomposition took place. When the cultures were aerated with air (21% oxygen), mycelial growth continued until the glucose was depleted and CO_2 production continued even past this point. Under 100% oxygen, the lignin polymer decomposition rate was two to three times faster than with 21% oxygen concentration. Mycelium growth was essentially negligible after the initial three days. This study links growth cessation with lignin decomposition, possibly indicating a switch to a secondary metabolism where substantial growth does not continue while the fungus breaks down lignin compounds.

If *Phanerochaete chrysosporium* degraded lignin better under 100% oxygen, a possibility existed that increasing the oxygen concentration by using hyperbaric systems would further improve the process as well (Reid and Seifert 1980). Pressure chambers with one to six atmospheres of applied oxygen were run and total lignin conversion by *Phanerochaete chrysosporium* measured. Lignin degradation was similar at one and two atmospheres of oxygen, but was inhibited at three atmospheres and completely stopped due to fungus death at four atmospheres and up. At five atmospheres of air, approximately equivalent to one atmosphere of oxygen in terms of oxygen concentration, lignin degradation occurred at the same rate as in one atmosphere of oxygen, showing

that oxygen concentration caused fungus toxicity and death, and that pressure was not a factor in the inhibition. An increase in oxygen concentration above 100% at one atmosphere was therefore not beneficial to fungal degradation of lignin.

100% oxygen addition was also shown to improve delignification of unbleached kraft pulp by *Phanerochaete chrysosporium* (Kirk and Yang 1979) and decomposition in still cultures (Yang *et al.* 1980). Lower oxygen levels, 7%, 14%, and 21% oxygen, were investigated in a factorial experiment measuring lignin biodegradation (Leszkiewicz and Kinner 1985). The highest concentration produced the strongest lignin degradation. Reid and Seifert (1981) showed that an oxygen atmosphere improved fungal delignification by almost two fold in agitated cultures of white-rot fungi, including *Phanerochaete chrysosporium.*

Decolorization of kraft bleach plant effluent with *Phanerochaete chrysosporium* was studied at five oxygen concentrations, ranging from 20 to 100% (Eaton *et al.* 1980). In stationary cultures, 80 and 100% oxygen concentrations improved the decolorization rate. However when the cultures were agitated, no significant difference was found among the different oxygen supplies. These findings suggest that oxygen diffusion may be a limiting factor rather than atmospheric oxygen concentrations.

Leisola *et al.* (1983b) discussed oxygen transfer during lignin degradation by *Phanerochaete chrysosporium.* Limited oxygen was available even under 100% oxygen atmospheres as shown by membrane electrode dissolved oxygen measurements just below the mycelial mat surface. Lignin degradation with air and oxygen atmospheres was found to be similar when the thickness of mycelial mats was reduced to 1 mm or less. Extreme variances between replicate runs were explained as a product of differing thicknesses of fungus permitting more or less oxygen transfer to occur.

Oxygen is crucial to lignin degradation or pulp mill effluent decolorization by *Phanerochaete chrysosporium.* Although some researchers have found that air supplies enough oxygen for the oxidative reactions, a 100% oxygen atmosphere or aeration has been used in most continuing work in order to maximize the reaction rate.

2.5.7 Agitation

Agitation of *Phanerochaete chrysosporium* influences two opposing factors. Improved oxygen transfer may result when a thick fungal biomass prevents efficient diffusion into interior cells. Agitation may also cause stress and shear on the biomass, thereby reducing its ability to degrade organics.

Kirk *et al.* (1978) tested lignin metabolism by *Phanerochaete chrysosporium* under six culture agitation conditions. Stationary cultures with 100% oxygen and 21% oxygen were paired with cultures under the same aeration, but that were shaken after nine days. Two other conditions used 21% and 100% oxygen concentrations where the cultures were shaken from the beginning of the test. The stationary 100% oxygen cultures outperformed all others by at least a factor of two. 100% oxygen in shaken cultures degraded more lignin than either 21% oxygen in agitated or stationary cultures, indicating that oxygen supply may have been more of a limiting factor than agitation. While cultures with 100% oxygen shaken from the start did not convert lignin to CO_2, 21% oxygen tests under continuous agitation did show some lignin biodegradation. The researchers observed that lignin conversion to CO_2 was proportional to the quantity of lignin not bound to the fungus pellets and that hyphae binding the lignin within the center of the biomass may have reduced oxygen availability at these sites.

Formation of spherical mycelium pellets during agitation was also found to reduce lignin degradation (Yang *et al.* 1980). Stationary cultures degraded lignin at least ten times faster than agitated cultures with both under 100% oxygen atmospheres. Although lignin biodegradation was inhibited, cellulose consumption in agitated cultures was 70% of that in stationary cultures.

In measuring by-products of lignin biodegradation, researchers have found similar effects of culture agitation. Veratryl alcohol is produced by *Phanerochaete chrysosporium* as a secondary metabolite during lignin biodegradation. Shimada *et al.* (1981) suggested a lignin degrading pathway that involved 3,4-dimethoxycinnamyl alcohol as an intermediate. This compound, added to *Phanerochaete chrysosporium* cultures, was converted to veratryl glycerol and veratryl alcohol under conditions previously used in lignin degradation. In contrast to stationary cultures, agitated cultures under 100% oxygen slowly metabolized the starting compound without producing veratryl alcohol which was present in static cultures. Because the system should not have been as sensitive to an oxygen gradient as in the lignin degradation experiments, the researchers

hypothesized that agitation may inhibit the secondary metabolism of *Phanerochaete chrysosporium.*

Ligninase activity was completely stopped during agitation of growing cultures (Faison and Kirk 1985) demonstrating a connection between liginase production and the more complex lignin biodegradation. Similarly, a stirred tank reactor was unsuccessful at producing liginase (Kirk *et al.* 1986) while reactors where agitation was minimized showed high levels of liginase present.

In bench scale RBC's, disc rotational speed affected decolorization of bleach plant effluent by *Phanerochaete chrysosporium* (Campbell *et al.* 1982). The MyCoR reactor showed the highest decolorization rates at 1 rpm disc speed with reduced rates at 7 and 18 rpm. Diameter of the discs in the MyCoR reactor is 18.4 cm. Shear and fungal sloughing apparently negated the expected improvements from higher oxygen transfer at greater disc velocities.

In contrast to these results, another set of researchers have conducted experiments that either show no adverse effects of agitation or show beneficial aspects.

Reid and Seifert (1981) used agitated *Phanerochaete chrysosporium* cultures to prevent oxygen depletion during lignin degradation without any visible negative effects. To further investigate this difference, Reid *et al.* (1985) compared agitated vs. static cultures with both synthetic and natural lignins. Using varying oxygen concentrations (21% and 100%) and stationary contrasted with agitated cultures as the test parameters, the research found that synthetic lignins were degraded at similar rates in both agitated and still cultures. While higher oxygen concentrations stimulated biodegradation in both culture sets, the shaken culture with an air atmosphere released more radioactive CO_2 from the labeled synthetic lignins. With natural aspen lignin, the shaken cultures produced more $^{14}CO_2$ under both oxygen concentrations than their stationary counterparts. Large mycelial pellets formed during agitation were compared to the mats formed in static systems. Smaller fragmented pellets similar to those reported by Kirk *et al.* (1978) in some agitated cultures showed slow metabolism of glucose and lignin.

Oxygen limitations may be negated in agitated cultures (Eaton *et al.* 1980), allowing decolorization of BKE to occur at the same rates under oxygen concentrations varying from 20% to 100%. The research used an initial stationary period of four days before

agitating the cultures. Although improved use of limited oxygen was found during agitation, poorer fungal growth resulted from mechanical stresses in an initial agitated growth period. Further decolorization tests with the already stressed fungus showed inhibitory effects on colour removal.

Different white-rot fungi were evaluated for enzyme production in shake flasks of E_1 stage BKE (Lankinen *et al.* 1991). *Phanerochaete chrysosporium* was found to produce both manganese and liginase peroxidases while the fungus achieved over 80% colour reduction in 15 days. In opposition to previous work (Faison and Kirk 1985 and Kirk *et al.* 1986), agitation did not inhibit enzyme production. Despite the high colour reductions achieved, Lankinen *et al.* (1991) did not have confidence in their LiP and MnP enzyme assay methods.

Other research on colour removal from BKE has shown no adverse effects of agitation. Prouty (1990) used *Phanerochaete chrysosporium* under a high aeration rate (700 to 6000 cm^3/min) which created an agitated environment in a 10 L Plexiglas cylinder reactor. At 4500 cm^3/min, over 1200 CU/(L•day) were removed from the effluent. Fukui *et al.* (1992) used shaken cultures to evaluate decolorization and detoxification of BKE by *Phanerochaete chrysosporium*. Over 65% colour removal, 57% AOX reduction, and an overall toxicity decrease were found in the cultures. Onset of secondary metabolism was observed after an initial four day growth stage, with a faster shaking speed (150 rpm, 25 mm radius) during the first two days than for the remainder of the experiment (120 rpm).

The obvious difference in results of agitation effects cannot be explained. Both sides have substantial research work measuring different parameters, including lignin biodegradation, enzyme production, and colour reduction to support their conclusions. Laboratory specific results are unusual, but small differences in procedures may have created the opposing results. As Reid *et al.* (1985) observed, fragmentation of the fungal biomass under some agitated conditions may cause inhibition of metabolic processes which are not seen to be limited under static or controlled agitation conditions.

2.5.8 Chemical culture conditions

P. chrysosporium requires a growth period before decolorization and ligninolytic activity occurs. Research to optimize the culture conditions during this period has focused on varying nutrient levels to achieve the highest lignin degradation rate.

Kirk *et al.* (1978) defined a simple medium for growth and lignin degradation based on measurements of CO_2 production from synthetic lignins under varying conditions. While the source of available nitrogen was not significant to lignin degradation, the quantity of nitrogen was critical to the timing of the lignin degradation onset. CO_2 production occurred at a much higher rate, even with reduced glucose consumption, when the culture was nitrogen limited. Thiamine was found to be the only vitamin required, even though a more complete mix encouraged better growth.

Nitrogen limitation may not be the only trigger for ligninolytic activity. Jeffries *et al.* (1981) found that carbohydrate and sulphur limitations could also activate the system. Nitrogen addition (as NH_4^+) was not an inhibitory factor when carbohydrates were already reduced.

Nitrogen limiting conditions may initiate the ligninolytic enzyme system of *P. chrysosporium* even without lignin present (Keyser *et al.* 1978), suggesting a non-specific pathway for lignin biodegradation. After nitrogen depletion typically occurred within the first 24 hours of growth, ligninolytic activity appeared within 72 to 96 hours. Further addition of ammonium before appearance of lignin degradation delayed its arrival and addition during the ligninolytic phase resulted in inhibition. These findings support the concept that secondary metabolism must be initiated before biodegradation occurs, a conclusion obtained by other researchers (Fenn and Kirk 1981).

Other research (Reid 1979) suggests that rather than individual concentration of carbon and nitrogen being important, the ratio of carbon:nitrogen was critical to controlling and predicting lignin degradation. Highest production of $^{14}CO_2$ occurred at a carbon to nitrogen ratio of 3355 on an carbon atom:nitrogen atom basis. Although a relationship existed between sulphate and nitrogen limitations, neither sulphate nor phosphate was found to suppress ligninolytic activity.

Up to 1.2% nitrogen added to TMP enhanced degradation while greater amounts were inhibitory (Yang *et al.* 1980). High levels of glucose were also found to react negatively with 1.2% nutrient nitrogen by suppressing lignin decomposition.

Faison and Kirk (1985) also found that ligninase is produced by nitrogen or carbohydrate starved cultures, again linking secondary metabolic events to lignin degradation.

Nitrogen may inhibit lignin metabolism because 1) growth rate and depletion of carbon source increases at higher nitrogen concentrations, 2) nitrogen and lignin metabolism cannot occur simultaneously, or 3) nitrogen controls the production of a necessary component in lignin degradation.

Interactions among various additives, including nitrogen, carbon, Tween 80 (a surfactant), and trace nutrients have been investigated in attempts to optimize decolorization or lignin degradation. Synergistic effects of nitrogen and minerals in combination with Tween 80 have been found to improve decolorization in the MyCoR reactor (Vasudevan *et al.* 1986). Mineral addition increased colour removal from 2700 CU/day up to 3800 to 4000 CU/day depending on the stage of addition. Tween 80 alone slightly improved decolorization; however, in conjunction with nitrogen, colour removal occurred at a slower rate. In combination with minerals, no difference was apparent. When Tween 80, minerals, and nitrogen were all added, the colour removal rate was increased to 7000 CU/day. Asther and Corrieu (1987) found that Tween 80 increased ligninase activity up to concentrations of 0.07% (w/v). Even higher ligninase activities were produced when cultures were supplemented with 0.04% oleic acid emulsified with Tween 80. One explanation for a surfactant's ability to improve decolorization and enzyme activity was suggested to involve modification of the fungus plasma membrane, permitting more efficient uptake and release of compounds through the cell wall.

Acclimatization has also been studied with relation to improving decolorization. Because pulp mill effluent contains many chemicals from the pulping or bleaching process that would not normally be found in a natural environment, the effluent may have toxic or inhibitory effects when introduced directly to the fungus. Fukui *et al.* (1992) evaluated decolorization after fungus pre-growth in various concentrations of E_1 effluent with all the necessary nutrients. Without E_1 effluent in the growth stage, decolorization was delayed by up to 5 days. The concentration of effluent added to the growth media had the same effect on subsequent decolorization regardless of the amount added between the ranges of 30 to 80%. Above 80%, growth and enzyme production are inhibited, possibly due to effluent toxicity.

2.5.9 Carbon requirements

Because lignin is such a complex polymer, *P. chrysosporium* and most, if not all other white-rot fungi, cannot degrade the polymer without a cosubstrate present (Kirk *et al.*

1976; Kirk *et al.* 1978; Reid 1979; Eaton *et al.* 1982; Kirk and Shimada 1985). In natural environments, the fungus produces cellubiose enzymes to break up the relatively simple cellulose into glucose molecules that can be used to further decompose the lignin. In environments where this natural cosubstrate is missing, such as in pulp mill effluent, an easily biodegradable carbon source is added to achieve biodegradation. While glucose is typically used in laboratory testing, other sources of carbon that can be used by *P. chrysosporium* are summarized in Table 2.6.

Table 2.6. Carbon sources that have been used in lignin degradation or pulp mill effluent decolorization studies with *P. chrysosporium*

Substrate	Reference
Glucose Glycerol Cellulose Sodium succinate	Kirk *et al.* 1978
Xylose Malate Mannose	Fenn and Kirk 1981
Pulp and paper mill primary sludge	Eaton *et al.* 1982
Karo corn syrup	Campbell *et al.* 1982

Kirk *et al.* (1978) speculated that despite lignin being a rich source of carbon and energy, not enough energy may be released during lignin metabolism to support growth and that ligninolytic activity may be too low to support growth. *P. chrysosporium* can produce glucan type polysaccharides under nitrogen limited conditions (Leisola *et al.* 1983a). Non agitated, air aerated cultures produced the highest non-glucose carbohydrate levels, up to 2.15 g/L after 15 days. When limited concentrations of glucose (2.4 g/L) were available, polysaccharide levels peaked at the glucose depletion point then began to decrease. The lower glucose levels achieved higher lignin degradation over the higher glucose level where increasing concentrations of non glucose carbohydrates may have inhibited biodegradation of complex compounds such as lignin. This research shows that only a reduced amount of easily biodegradable substrate may be needed because the fungus can produce simpler carbohydrates from lignins. Kirk and Shimada (1985) point out a balance between energy consumption and energy production governs the need for a co-substrate during lignin degradation.

Glucose has been used in most laboratory evaluations of *P. chrysosporium* decolorization of pulp mill effluent. High concentrations, such as 1%, would be expensive in any larger scale system and would cause undesirable increases in BOD and COD of the effluent. A critical glucose concentration of between 1050 and 2100 mg/L was needed to sustain decolorization activity in a one day batch MyCoR reactor (Yin *et al.* 1989b). Higher glucose levels did not enhance colour removal. Of this reduced amount, the fungus only consumed less than a third, leaving the remainder in the effluent. As this easily biodegradable substrate would cause significant oxygen depletion in receiving waters, carbon utilization in the fungal process in an important consideration in any future work.

Recent work has looked at increasing the available carbon through ozonation (Mao 1996). Ozonation of previously biologically treated effluent has been shown to increase the BOD and improve its biotreatability (Heinzle *et al.* 1992; Mohammed and Smith 1992; Mao and Smith 1995; Roy-Arcand and Archibald 1995). Although most decolorization work with *P. chrysosporium* has used alkaline extraction effluent where the colour is most concentrated, the reduced colour removal rate by *P. chrysosporium* in ASB effluent may be offset by lower additional carbon requirements. Combining ozone and fungal treatment with *T. versicolor*, Roy-Arcand*et al.* (1991) found improved decolorization and TOC removal in E_1 effluent. An additive effect on AOX removal was found where ozone and the fungus may degrade different portions of the total AOX. Mao (1996) used combined ASB effluent to study possible synergistic effects of ozone and *P. chrysosporium* on colour and AOX removals. Some evidence presented suggests that the additional carbon substrate required in other studies may be eliminated with pre-ozonation, vastly improving the economic and environmental feasibility of the project. Further work still remains to verify these results and to apply them in an engineered system.

Table 2.7 summarizes the conditions most favorable to *P. chrysosporium* growth and decolorization. While some research has yielded results contradictory to those outlined here, the general consensus on cultural parameters is presented.

Table 2.7. Conditions most favorable to growth and decolorization by *P. chrysosporium*

Parameter	Condition
pH	4.0 to 5.0 for growth and decolorization.
Temperature	35 to 40°C
Oxygen requirements	Air or full oxygen atmosphere required depending on transfer efficiency.
Agitation	Uncertain.
Detention time	Long detention times normal, but some systems require only 1 day.
Carbon requirements	Co-substrate necessary. 1050 to 2000 mg/L glucose minimum concentration.
Initial colour	Higher removal rate at higher colours. Initial colour > 6000 CU
Other	Nitrogen inhibits decolorization. Tween 80 improves decolorization even with nitrogen present. High levels of chlorides do not interfere with decolorization.

2.5.10 Metabolic pathways and enzyme production

The enzyme system of *P. chrysosporium* activated to degrade lignin has been studied to learn the fundamental pathways of the process, to investigate how best to optimize lignin degradation, and to isolate the enzymes for possible synthesis and use in industry. Other reviews of *P. chrysosporium* 's metabolic pathways and enzymes produced are included in Reid (1994), Eriksson (1990), and Kirk and Shimada (1985).

Natural and chlorinated lignins may differ in their aqueous solubility, but the same extracellular enzymes are responsible for the degradation of both compounds. Non-specific mechanisms are used by white-rot fungi, i.e. the enzyme production is not triggered by the presence of a specific chemical. Both lignin and modified lignins are high molecular weight polymers with many branching functional groups and would not be easily taken in through the fungal cell wall regardless of solubility. Extracellular enzymes are capable of fragmenting the large compounds which can then be taken through the cell membrane for further degradation ending in CO_2 release (Reid 1994).

The term "enzymatic combustion" has been used to describe how enzymes initiate lignin to begin oxidatively fragmenting the molecule without enzymatic pathway control (Kirk and Farrell 1987). This process may account for why polymerization does not reoccur as expected with oxidation of lignins containing free phenolic groups.

Lignin degrading enzymes are still being studied to identify their role in lignin degradation and the conditions that activate them. In *P. chrysosporium* cultures, at least two oxidative and one reductive enzyme are typically found when biodegradation is taking place. Other fungi use these, as well as other enzymes, to achieve the same final conversion (Odier and Artaud 1992).

2.5.10.1 Ligninase or lignin peroxidase (LiP)

Ligninase is a true peroxidase and iron-hemeprotein (Odier and Artaud 1992) that was discovered based on the peroxide dependent C_α-C_β cleavage of synthetic lignin compounds (Cullen and Kersten 1992). LiPs, having multiple forms, range in molecular weight from 38000 to 43000 and have a low optimum pH around 2 (Boominathan and Reddy 1992 and Odier and Artaud 1992). LiPs act through six main mechanisms to degrade compounds: 1) bezylic alcohol oxidation, 2) carbon-carbon bond cleavage, 3) hydroxylation, 4) phenol dimerization or polymerization, 5) demethylation, and 6) aromatic ring cleavage (Boominathan and Reddy 1992). Removal of one electron from non-phenolic aromatic substrates is key to the reactions LiP initiates even with non lignin molecules (Barr and Aust 1994). LiP may not require direct contact with lignin during degradation of natural substrates (Garcia *et al.* 1987), as lignin was found to be degraded some distance from the fungal hyphae and LiP was found localized inside the cell wall. Veratryl alcohol was found to induce ligninase production (Faison and Kirk 1985 and Kirk *et al.* 1986) and to be a secondary metabolite of lignin degradation (Shimada *et al.* 1981). LiPs may play a minor role in pulp mill effluent decolorization, as seen in cultures with high LiP activity, but low MnP activity occurred where decolorization was lower compared with high MnP cultures which had high colour removals (Michel *et al.* 1991).

2.5.10.2 Manganese peroxidase (MnP)

Manganese peroxidase is another true peroxidase hemeprotein with molecular weight approximately 46000 and optimum pH at 4.5 (Kuwahara *et al.* 1984). MnPs function

primarily to oxidize Mn^{2+} to Mn^{3+} with H_2O_2 as an oxidant (Cullen and Kersten 1992) thus allowing the Mn^{3+} to oxidize other molecules, mainly phenolic substrates. Unlike LiP, MnP oxidizes NAD(P)H, creating peroxide with oxygen consumption. In *P. chrysosporium* cultures, the MnP enzyme was found in peripheral areas of the fungal hyphae on the cell membrane, on membranes of structures similar to vesicules, and on the cell wall (Daniel *et al.* 1990). MnPs are important in decolorization of bleach plant effluent by *P. chrysosporium* and their activity is enhanced with increased concentrations of Mn^{2+} in the medium (Michel *et al.* 1991).

2.5.10.3 Cellobiose: quinone oxidoreductase (CBQase)

CBQase is a flavoprotein with molecular weight of approximately 58000 whose synthesis is regulated along with cellulolytic enzymes (Kirk and Shimada 1985). This enzyme may prevent lignin repolymerization by reducing the phenoxy radicals formed as products of lignin biodegradation (Ander *et al.* 1990), but recent research contradicts this hypothesis (Odier and Artaud 1992). Extracellular quinone reductase may also be involved in maintaining a hydroquinone reserve for MnP catalyzed reductions (Barr and Aust 1994), which could shift the polymerization/depolymerization LiP reaction towards degradation by rapid reduction and metabolism of quinone compounds (Ander *et al.* 1990).

Barr and Aust (1994) presented reduction mechanisms for highly oxidized compounds based on the two main peroxidases, LiP and MnP. Veratryl alcohol cation radicals can oxidize certain chemicals by one electron, forming another radical that results in reduction. Organic acids, such as oxalic acid, were proposed as the electron donor the LiP dependent reductive pathway of *P. chrysosporium*. The proposed mechanism for MnP catalyzed reductions involves oxidation of hydroquinones to semiquinone radicals which then behave as reducing agents. These pathways may reduce the importance of the reductive mechanisms proposed for CBQase.

2.5.10.4 Other enzymes

While many other white-rot fungi produce laccase, *P. chrysosporium* has shown no evidence of this enzyme (Odier and Artaud 1992). Enzymes responsible for the utilization of the co-substrate in lignin biodegradation include glyoxal oxidase, glucose oxidases, aryl methoxyl demethylase (demethoxylase), methanol oxidase, β-glucanases (both endo and exo), and β-glucosidases. The first four enzymes have been suggested as

H_2O_2 sources (Reid 1994). Although H_2O_2 radicals are not directly involved in lignin degradation, they have been shown to have a temporal correlation with lignin degrading enzymes (Faison and Kirk 1985) and ligninolytic activity is suppressed when specific scavengers of H_2O_2, such as catalase, are introduced. β-glucanases are responsible for conversion of cellulose to glucose and cellobiose units while glucosidases hydrolyze cellobiose to glucose and cellobionic acid to glucose and gluconolactone (Cullen and Kersten 1992).

Intracellular enzymes most likely provide the final degradation of low molecular weight lignin fragments to CO_2 and accumulating byproducts. Insufficient characterization of these enzymes has been done to date. Some of the low molecular weight aliphatic and aromatic byproducts of lignin degradation have been determined, partly to better understand the pathways involved in their production. Extracellular and cell bound non-glucose polysaccharides have been found in *P. chrysosporium* cultures (Leisola *et al.* 1982). Veratryl alcohol is a known secondary metabolite of lignin degradation that may be synthesized from glucose or lignin with phenylalanine, veratrylglycerol, and 3,4-dimethoxycinnamyl alcohol as intermediates (Fenn and Kirk 1981). Oxalic acid is secreted by *P. chrysosporium* during lignin degradation (Barr and Aust 1994). Lignins modified during the bleaching process yield some different metabolic products because they are organically bound with chlorine. Major products of dechlorination of E_1 effluent included veratryl alcohol, veratraldehyde, vanillin alcohol, 4, 5-dichloroveratrole, 6-chlorveratryl alcohol, and 3,4-dimethoxyacetophenone (Huynh *et al.* 1985). Low molecular weight chlorinated phenols and other similar components were removed during the fungal treatment process. Inorganic chloride and nonaromatic organics were suspected to be predominant in dechlorination of high molecular weight chlorolignins by *P. chrysosporium* (Pellinen *et al.* 1988b).

3. MATERIALS AND METHODS

3.1 Analysis Methods

3.1.1 Wastewater samples and analysis

Pulp mill wastewater was obtained from Weyerhaeuser Canada Ltd., Grande Prairie Operations. Grab samples of ASB wastewater, generally from the second cell of the basin, were shipped in sealed 20 L containers then stored at 4 °C for up to 4 months. In each set of tests, the raw wastewater was analyzed along with the ozonated samples to detect any changes in the raw sample.

The ASB samples obtained in June, 1995 were less concentrated than other samples because the yearly maintenance shut down had occurred in May, 1995 with large volumes of wash water being drained into the basins. After normal operations resumed, some time was necessary before the effluent characteristics became stable.

Ozonated samples were stored in 4 L brown opaque glass bottles at 4 °C for up to one month before use. Generally ozonated samples were used in tests within one to two weeks after ozonation. If necessary for the set of tests being run, the pH of the ozonated wastewater would be adjusted to 4.5 with concentrated HCl and NaOH or potassium pthalate and NaOH buffer before storage. BOD change was negligible in the scope of the tests when the pH of the samples had been adjusted down to approximately 4.5 and when the samples were stored at 4 °C.

3.1.2 Ozonation of samples

A schematic of the bubble column used for ozonation is shown in Figure 3.1. A fine gas diffuser consisting of a spherical 25 mm crystalline aluminum stone was set 5 mm above the bottom of a Plexiglas column 100 mm in diameter and 1750 mm high, providing a typical batch volume of 8 L with sufficient head space remaining at the top for foam development. All materials, except for the diffuser, were made of Plexiglas, stainless steel, or Teflon to reduce ozone deterioration. Room temperature (20 to 22 °C) wastewater was pumped into the column from the bottom with a peristaltic pump. Ozone gas, generated from extra-dry oxygen by a corona discharge generator, was bubbled through the diffuser into the wastewater. Ozone concentration was monitored frequently to record any fluctuations that would affect the total dose. Typical experimental conditions consisted of about 80 mg/L ozone over a 30 minute period at a flow rate of approximately 1000 mL/minute of oxygen. Exhaust gas was collected and determined by the KI method

(Standard Methods 1989), which involves reacting the ozone into 400 mL of 2% KI solution then titrating with 0.2 N sodium thiosulphate solution. Exhaust gas was often negligible under normal operating runs of 8 L and full strength wastewater.

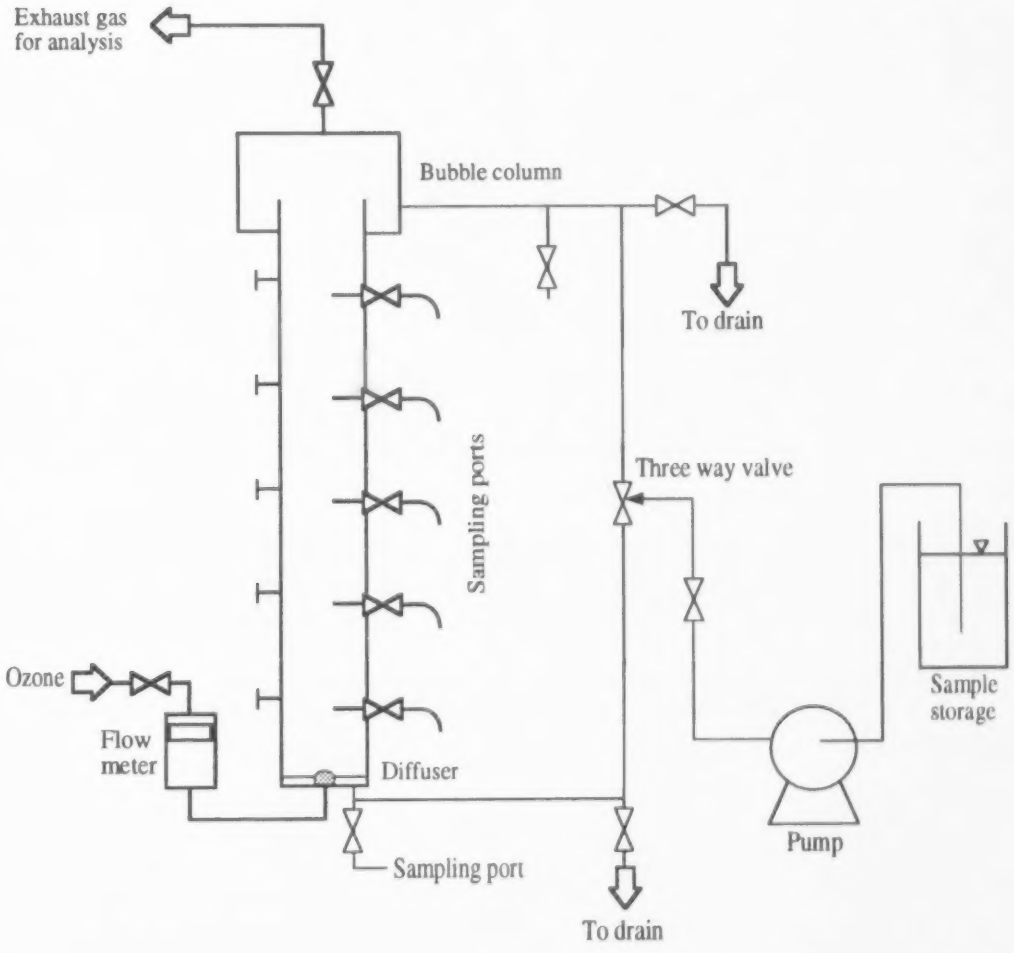


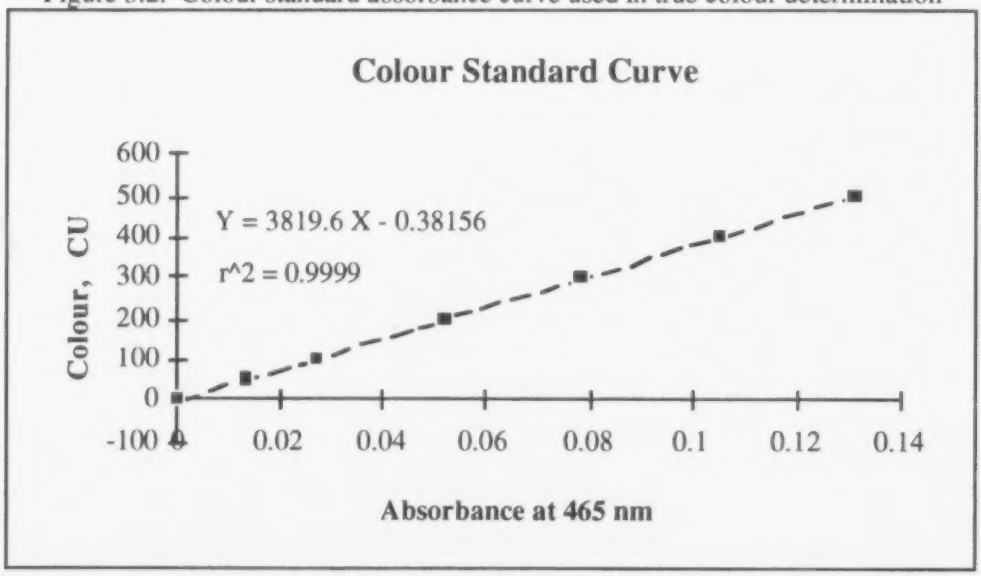
Figure 3.1. Schematic of bubble column ozonation system. Adapted from Zhou and Smith (1995).

3.1.3 True colour determination

The Canadian Pulp and Paper Association standard method H5.P (CPPA 1974) was used to determine true colour of the wastewater samples. A standard curve, shown in Figure 3.2, run in the range of 50 to 500 CU, was used to determine sample colour with a linear regression model. After any necessary dilutions with Milli-Q water, the pH of the samples was measured with a Fisher Accumet ® pH meter 25 and adjusted by the addition of 0.1 M NaOH and HCl to 7.6 ± 0.1 units. Samples were filtered through 0.8 µm MSI Micron Separations Inc. filters to remove suspended particles. The absorbance of the sample was measured in a 10 mm path length quartz cuvette with a Spectronic 601 (Milton Roy Company) at a wavelength of 465 nm. Using the regression equation calculated from the standards, the colour was determined and rounded off to the nearest 10 CU.

Modifications were made for the bioreactor samples in addition to the standard method of colour analysis. The pH of two samples of approximately 40 mL each was adjusted to less than 2.5 with the addition of HCl. The samples were then centrifuged at 3000 rpm for 45 minutes to settle any solids that may have precipitated at a low pH. One sample had its pH adjusted back upwards to 7.6 ± 0.1, while the pH of the remaining sample was left below 2.5 units. Both samples were then filtered and the absorbance determined as described above.

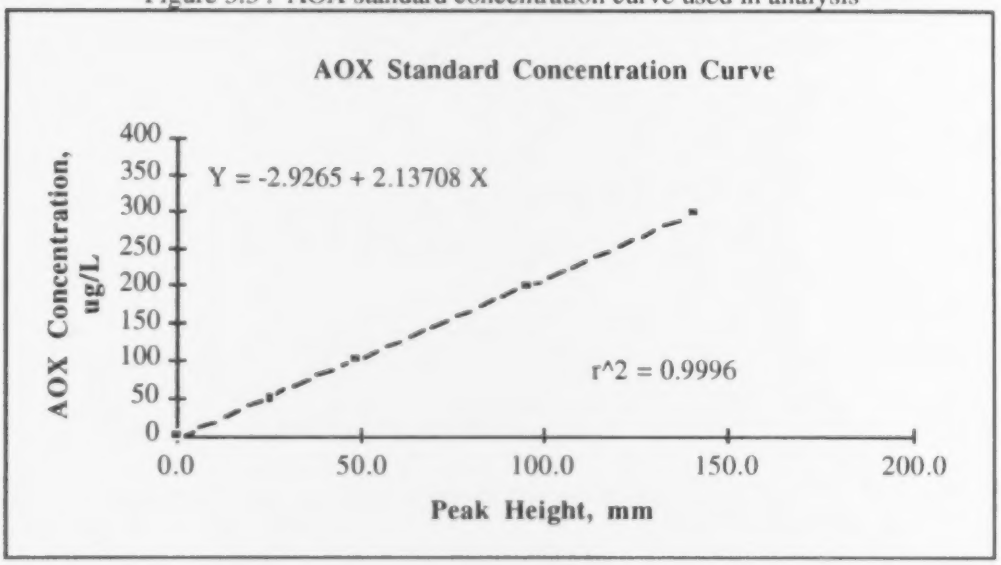
Figure 3.2. Colour standard absorbance curve used in true colour determination



3.1.4 Total and adsorbable organic halogen determination

Total and adsorbable organic halogens (TOX and AOX) were determined as approved by Standard Methods (APHA 1989). Samples were run through two columns of powdered activated carbon (PAC) to trap the organic halogens. The PAC was incinerated in a Euroglass Delft Holland AOX Analyzer and the concentration of organic halogens compared to a standard curve created with *p*-chlorophenol standards ranging from the blank to 250 µg/L AOX. Each sample was diluted to be within this range before analysis. A typical standard curve using *p*-chlorophenol is shown in Figure 3.3.

Figure 3.3 . AOX standard concentration curve used in analysis



3.1.5 Chemical oxygen demand determination

Standard Methods (APHA 1989) was followed in determination of the chemical oxygen demand (COD) of the samples (Method 508A, Dichromate Reflux Method), with mercuric sulphate added to the sample before the addition of other reagents to reduce chloride ion interference. Appropriate dilutions were made before analysis to avoid excess use of titrant. All COD analyses were done in duplicate samples.

3.1.6 Biochemical oxygen demand analysis

Pulp mill effluent acclimatized seed was used to measure the oxygen uptake and biodegradability of the raw and ozonated wastewater samples following method 507, Oxygen Demand (Biochemical) in Standards Methods (APHA 1989). The membrane electrode method (421 F) described in Standard Methods was used because interferences, presumably from the highly coloured effluent, created errors in the Winkler titration method. In the BOD bottles, 2 mL/L of seed, 30 mL of sample, and dilution water were added before the initial dissolved oxygen measurement. After five days incubation at 20 ± 2 °C, the dissolved oxygen in the samples were determined and the BOD_5 was calculated. Freshly ozonated samples were generally refrigerated for one day before BOD analysis to allow the oxygen levels to stabilize because the much higher oxygen levels in the sample would have introduced serious errors into the BOD measurement. All BOD samples were done in duplicate or triplicate with blanks run for each set.

3.1.7 Total organic and inorganic carbon determination

Total carbon (TC) was analyzed using the furnace of the Total Organic Carbon Analyzer (Dohrman Xertex Carbon Analyzer) at 850 °C. Method 505, Combustion-Infrared Method, was used according to Standard Methods (APHA 1989). Appropriate standards of potassium pthalate solutions were run to calibrate the instrument within the expected range of the samples. Dilutions of the samples were made, when necessary, to bring the sample into range of the standards. Inorganic carbon was analyzed by direct injection methods. The sample peaks were compared to the standard sodium carbonate (Na_2CO_3) for determination of total inorganic carbon (TIC). Total organic carbon was calculated by subtracting the TIC from the TC.

3.1.8 Total protein assay

The coomassie blue reaction method, as described by Sedmak and Grossberg (1977), was used to assess total protein present in the biologically treated pulp mill effluent. This procedure is typically more sensitive and less subject to interference by other compounds than the Lowry reaction. The assay works on the principle that coomassie brilliant blue G dye changes from red to blue when amino acid groups (proteins) bind to the dye.

Coomassie blue was prepared fresh before each set of tests by dissolving 100 mg of Coomassie brilliant blue G250 in 50 mL of 95% ethanol. 100 mL of 85% (w/v) of

phosphoric acid was added and the volume brought up to 1 L with deionized water. Protein standards were prepared from Sigma protein standard in the range of 0 to 400 µg/mL. Deionized water plus 2.5 mL dye solution served as the standard blank. In a clean test tube, 0.1 mL of the protein standard or the sample was mixed with 2.5 mL of the dye reagent and incubated for two minutes. Absorbance was read at 595 nm on the Bausch and Lauomb Spectronic 21 and compared to the standard curve to determine total protein present.

3.1.9 Microbiological work

3.1.9.1 Heterotrophic plate counts

Heterotrophic plate counts (HPCs) were done with membrane filtration through sterile 0.45 µm filters at various dilutions according to Standard Method 907 (APHA 1989), with triplicate samples at each dilution. The filters were placed on HPC media and incubated at 20 °C for seven days for one set, and at 35 °C for 48 hours for another set. Colonies were counted and the dilutions noted. The dilution that yielded approximately 20 to 80 colonies was considered the most accurate representation of the concentration of colony forming units in the sample. If excessive spreader growth, greater than 50% of the area available on the plate, occurs, the result was reported as "spreader" and no colony count was taken. A geometric mean was calculated to give an estimate of the actual population density in the sample being measured.

3.1.9.2 Yeast and fungi determination

Bioreactor populations were monitored with swabs taken from the surface of the sponges. Different media plates, including rose bengal, malt, and benomyl agar, were streaked with the swab and incubated at 35°C. Plates were checked after two to four days for microbial growth and select colonies were isolated for identification. Although this method did not allow a numerical population count, it did allow an assessment of the general microbial community.

3.2 *Phanerochaete chrysosporium* culture and preservation

Three strains of the white-rot fungus *Phanerochaete chrysosporium* were used in this research. One strain, ATCC 24725 was obtained from the American Type Culture Collection laboratories and transferred to slants for storage. The other two strains, UAMH

4521 and UAMH 3642, were acquired from the University of Alberta Microfungus Collection at the Devonian Botanical Gardens in Devon, Alberta. Table 3.1 presents information on the three strains of *Phanerochaete chrysosporium* .

Table 3.1. Information on three strains of *Phanerochaete chrysosporium* used in batch and bioreactor testing

	UAMH 3642	UAMH 4521	ATCC 24725
Isolation data	deadwood ex *Platanus wrightii*, Arizona sycamore Sonoran Desert, Cochise Colorado H. Burdsall 25-Aug.-71	ex *Vitis sp.* A.U.C. of Micro-Org., Alma-ata, Kazachstan, USSR 26-Mar-91	*Vitis vinifera*
Sender	W.E. Eslyn HHB 6251	H. Burdsall BKM-F-1767	L.A. Beljakova VKM F-1767, T. I. Novobranova
Strain Characteristics	Delignification of unbleached kraft pulp, veratryl alcohol production	biodeterioration/ biodegradation, cellulolytic, thermotolerant	Degradation of lignin, aromatic compounds, produces liginase and peroxidase
Cross Reference	ATCC 34540	IMI 174727; ATCC 24725; CBS 481.73	CBS 481.73; CMI 174727; NRRL 6361; UAMH 4521

Three strains of *P. chrysosporium*, designated by their culture collection numbers, were evaluated in the batch tests. UAMH 4521 and ATCC 24725 may be the same strain since the UAMH 4521 is cross-referenced with the ATCC 24725. These two also display similar growth patterns, conidial development, and performance in decolorization of pulp mill effluent. UAMH 3642 has distinct spore formation which sets it apart from the other two strains tested.

3.2.1 Storage and transfer of *Phanerochaete chrysosporium*

Cultures were stored on slants of cereal or malt agar at 4 °C and transferred periodically to maintain culture viability.

To transfer the fungus strains for use in laboratory experiments, cereal agar plates were inoculated from the slants under aseptic conditions. Cereal agar was made by combining

100 g/L baby food cereal, 15 g/L bacto-agar, and deionized water. The mixture was autoclaved at 121 °C for 30 minutes, cooled slightly, then poured into disposable petri plates. Cereal agar plates also served as starters for new plates when small blocks would be cut from the agar and placed fungus side down on the sterile media. In most experiments, the plates were allowed to grow for three weeks at 35 °C before they were stored in an incubator at 20 °C until used. The long incubation time allowed a thick layer of spores to develop which could then be scraped off easily for culture in a different medium.

In batch experiments requiring large masses of fungus, plates were started as described above, but only incubated for one week at 35 °C before use. The shorter incubation time did not allow substantial spore formation; rather the fungus was still in an active growing stage with mostly mycelium scraped or washed off the plates to begin the cultures.

3.2.2 Nutrient solution preparation

A growth solution was used to culture the fungus from the agar plates to the liquid wastewater. The solution was made up in 6 to 12 L batches, poured into 1 L flasks, autoclaved at 121 °C for 25 to 40 minutes, and stored at 4 °C until needed. Nutrient and mineral concentrations, as listed in Table 3.2, were based on growth solutions first discussed by Kirk *et al.* (1978) as necessary for *Phanerochaete chrysosporium* growth.

In replication of work by Mao (1996), an "improved medium" was added to the wastewater to test if it enhanced growth and decolorization by the fungus. A different buffer, potassium pthalate, was used instead of 2,2 dimethyl succinate because the latter was not readily available. The previous research work had used the improved medium in liquid form prepared in 50 times the regular concentration and diluted to the desired amount in the wastewater. In this work, because the mineral solids and buffer could not be dissolved to make a 50 times concentration, the desired amounts were added directly in solid form to the wastewater and mixed until dissolved. The improved medium and the basal medium are detailed in Table 3.3.

Table 3.2. Nutrient and mineral solutions used to culture *Phanerochaete chrysosporium* for decolorization and growth studies

Nutrient solution		Mineral Solution	
Compound	Concentration, mg/L	Compound	Concentration mg/L
Glucose	10 000	$MgSO_4 \cdot 7H_2O$	3 000
NH_4Cl	11.8	$MnSO_4 \cdot H_2O$	500
KH_2PO_4	200	NaCl	1 000
$MgSO_4 \cdot 7H_2O$	50	$FeSo_4 \cdot 7H_2O$	100
$CaCl_2 \cdot 2H_2O$	13	$CaSO_4 \cdot 7H_2O$	181
Thiamine HCl	0.1	$CaCl_2 \cdot 2H_2O$	109
Mineral Solution	1.0 mL/L	$ZnSO_4 \cdot 7H_2O$	178
		$CuSO_4 \cdot 5H_2O$	10
		$NaMoO_4$	10

Table 3.3. Composition of improved and basal medium . After Mao (1996).

Basal medium		Improved medium	
Compound	Concentration, mM	Compound	Concentration mM
Potassium pthalate*	25	Basal Medium	as given
NaOH*	4	$MnSO_4 \cdot H_2O$	0.5 mM
KH_2PO_4	1.47	Thiamine•HCl	0.1 mg/L
$MgSO_4 \cdot 7H_2O$	0.21	Mineral solution	1 mL/L
$CaCl_2 \cdot 2H_2O$	0.09	Tween 80	0.05%

* Substituted for 2, 2-dimethylsuccinate

3.2.3 Spore suspension preparation

A spore suspension was prepared according to Kirk *et al.* (1978) to standardize the spore concentration used to start the batch experiments. Under aseptic conditions, sterile nutrient solution was added to the surface of 3 to 4 weeks old spore plates and the aerial spore growth gently scraped off to remove the spores without scraping into the agar. The spores were then mixed with nutrient solution, as described previously, and 0.1 mL Tween 80 per 100 mL of solution until a mixed suspension was obtained. Sterile water was not used because the high reactivity of the water may have had an effect on the viability of the

spores. A small volume of the suspension was withdrawn and the absorbance at 650 nm measured. This volume was diluted, in successive steps until the absorbance was 0.5 on the Spectronic 21. This absorbance is equivalent to approximately 2.5×10^6 spores per mL (Kirk *et al.* 1978). The original spore concentration could then be determined and adjusted with the addition of sterile nutrient solution. An aliquot of the spore suspension was then pipetted into each flask, with mixing in between each withdrawal to ensure relatively uniform concentration of spores.

Age of the plates, humidity, and strain of fungus were all found to influence the apparent spore concentration. The older, drier plates had much higher absorbance readings than the younger plates that had been kept in a tightly sealed bag throughout their incubation for the same quantity of plates in the same amount of solution. Both ATCC 24725 and UAMH 4521 produced thicker layers of conidia and mycelium than UAMH 3642 did. However, the absorbance of the spore suspension indicates nothing about viability of the spores and may be affected by the amount of extra mycelium that had already ceased to grow.

3.3 Immobilized fungi rotating biological contactor (IF-RBC) tests

3.3.1 Bioreactor design

Four bioreactors were constructed in the Engineering Machine Shop at the University of Alberta from standard stock 304 grade 16 gauge stainless steel. Plexiglas lids were constructed to cover the reactors and minimize both airborne contamination and evaporation losses. Figures 3.4 to 3.5 show schematics of the bioreactor. The new bioreactors were constructed from stainless steel to be autoclavable, temperature resistant, and durable. Evaporation control and contamination prevention were major considerations in the reactor design. Dye tests were conducted on one new reactor to determine the flow regime. These results are shown in Appendix C. In summary, when the reactors were in operation with slow rotation of the foam sponges, neither plug flow nor completely mixed flow dominated. Rather, arbitrary flow was present where longitudinal dispersion did occur, causing the tracer dye to be found in advance of the high concentration front. Similar flow patterns were seen at both 1 and 2 rpm when the dilution factor during the second test was considered. Sponge rotation may have caused higher mixing, closer to completely mixed conditions, in each individual cell of the reactor with slower mixing across the cell dividers. As these tracer tests should have been carried out longer, much of the information on reactor flow regime is missing.

Each reactor was divided into four cells by semi-circular steel plates that fit into slots on the reactor body. Short circuiting was prevented by alternating which side the effluent could flow through a circular hole near the bottom of one side of each divider. Flow in the cells was along the bottom while the final effluent was collected over the top of the final fixed partition into a non-aerated zone and out the effluent pipe. The reactors were modified to have curved tubing airlines that were held in place at the beginning of each cell by a short section of metal tubing welded to the reactor.

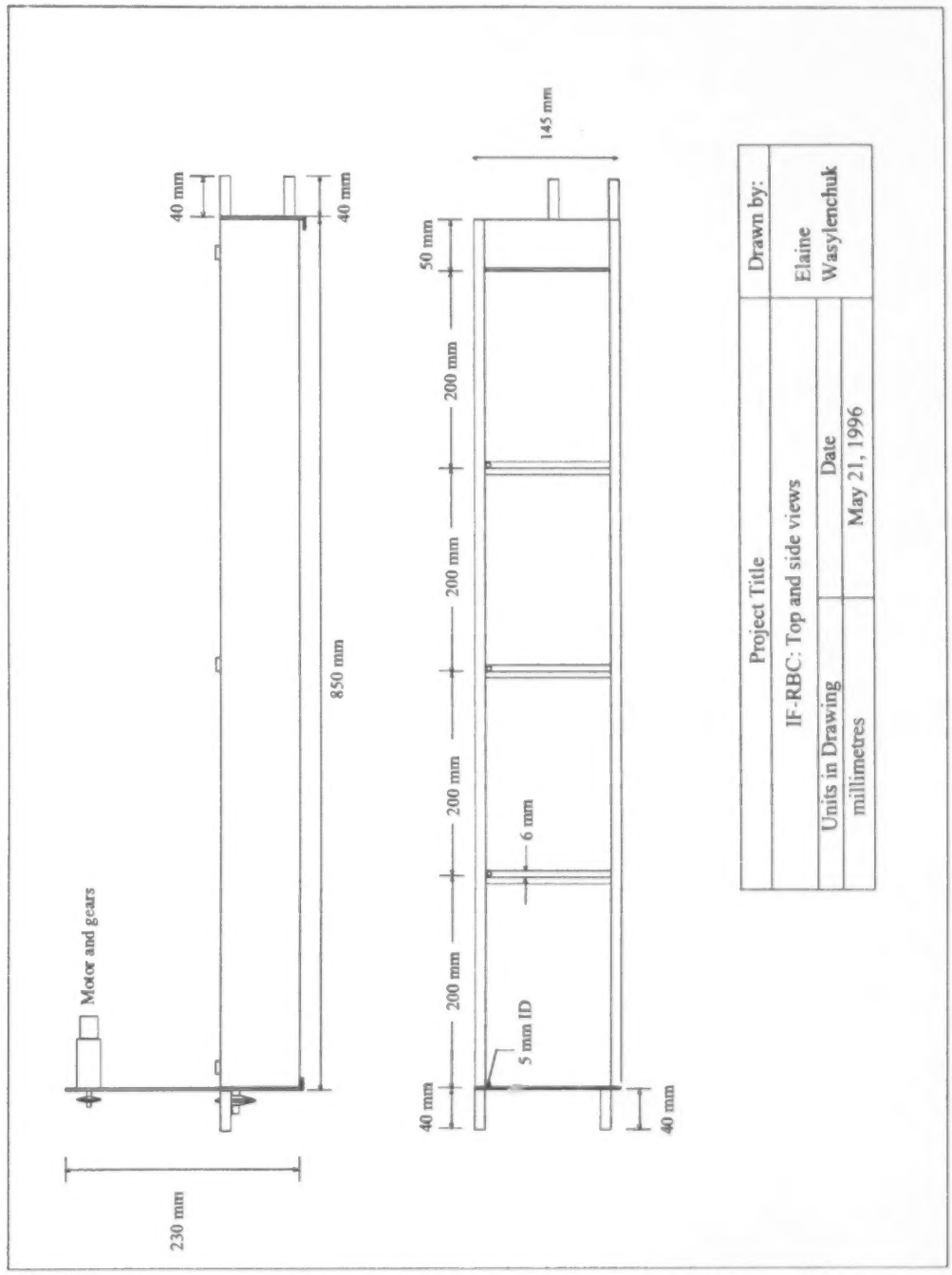


Figure 3.4. Immobilized fungi rotating biological contactor: top and side views of bioreactor showing reactor partitions, influent and effluent ports, and overflow zone.

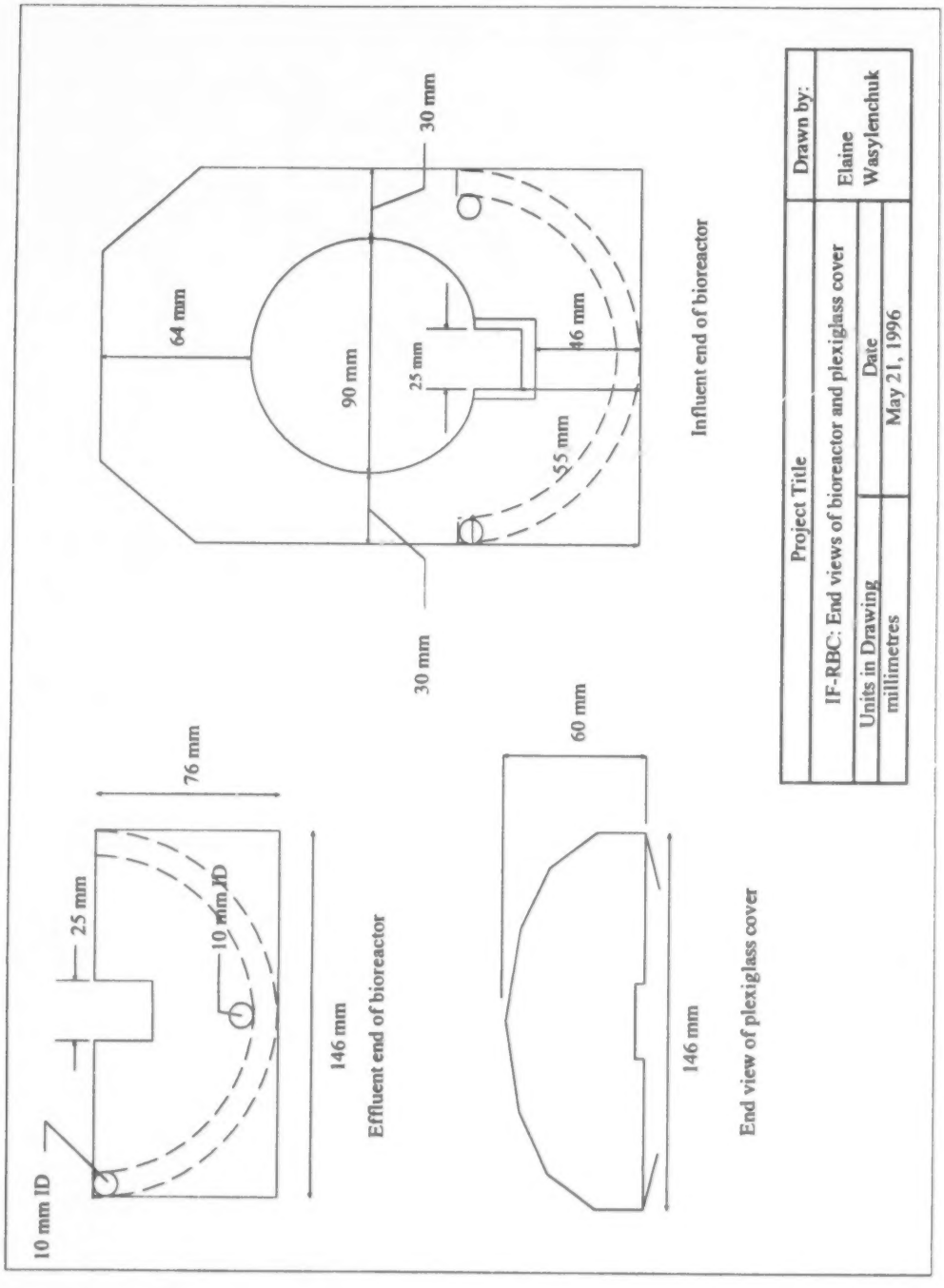


Figure 3.5. Immobilized fungi rotating biological contactor: end views of bioreactor and Plexiglas cover.

Reactor temperature was maintained by the water bath built around the reactor. When three reactors were run, a circuit was set up; from the hot water bath (Blue M Magni Whirl constant temperature bath), water was pumped into the first reactor, through the remaining two, and back into the water bath to be reheated. The pump (Haake D1 pump and heater) provided sufficient recirculation that the temperature difference among the three reactors was never greater than 1°C. Figure 3.6 shows a schematic of the IF-RBC set up in the laboratory with all three reactors running.

Steel shafts held the sponges in place while the Pittman GM9413-5 motor (12VDC, 728:1 ratio) rotated the shaft. A dual regulated DC power supply (Anatek Model 25-20) allowed the shaft rotation speed to be adjusted. Sponges were separated into each cell by steel discs, 52 mm in diameter that could be tightened to the shaft with set screws to prevent the sponges from sliding.

Plexiglas covers were constructed to minimize evaporation, air borne contamination, and oxygen losses. The long sides of the lids were modified to reduce evaporation further, by creating a drip ledge for condensation to run back into the reactor. Ribbed rubber tape weather-stripping was also run along the drip ledge to better seal the reactors.

Extra-dry oxygen was used to aerate the reactors. An air flow splitter ran from the regulator on the oxygen tank to the gang valves that provided aeration for each cell. Flow rate and pressure were adjusted as necessary, depending on the number of reactors being used at once, to maintain dissolved oxygen concentrations near saturation.

Effluent was fed to the reactors from a storage reservoir at 4°C by pumps (Cole-Parmer Masterflex computerized drive, Masterflex Cole-Parmer 7567-50, and Cole-Parmer 7553-80, all with 7016 pump heads). Controllers for two pumps were Masterflex solid state speed controls (Model 7553-71). The third pump had its own computerized controller. The three pumps allowed different flow rates to be set for each reactor.

Because the first reactor built had a slightly shorter overflow weir than present in the other two reactors, the reactor volumes were different and had to be taken into account when determining a flow rate necessary to achieve a given hydraulic retention time. Reactor #1 had a working volume (with sponges in reactor) of 3660 mL while reactors #2 and #3 had volumes of approximately 4000 mL.

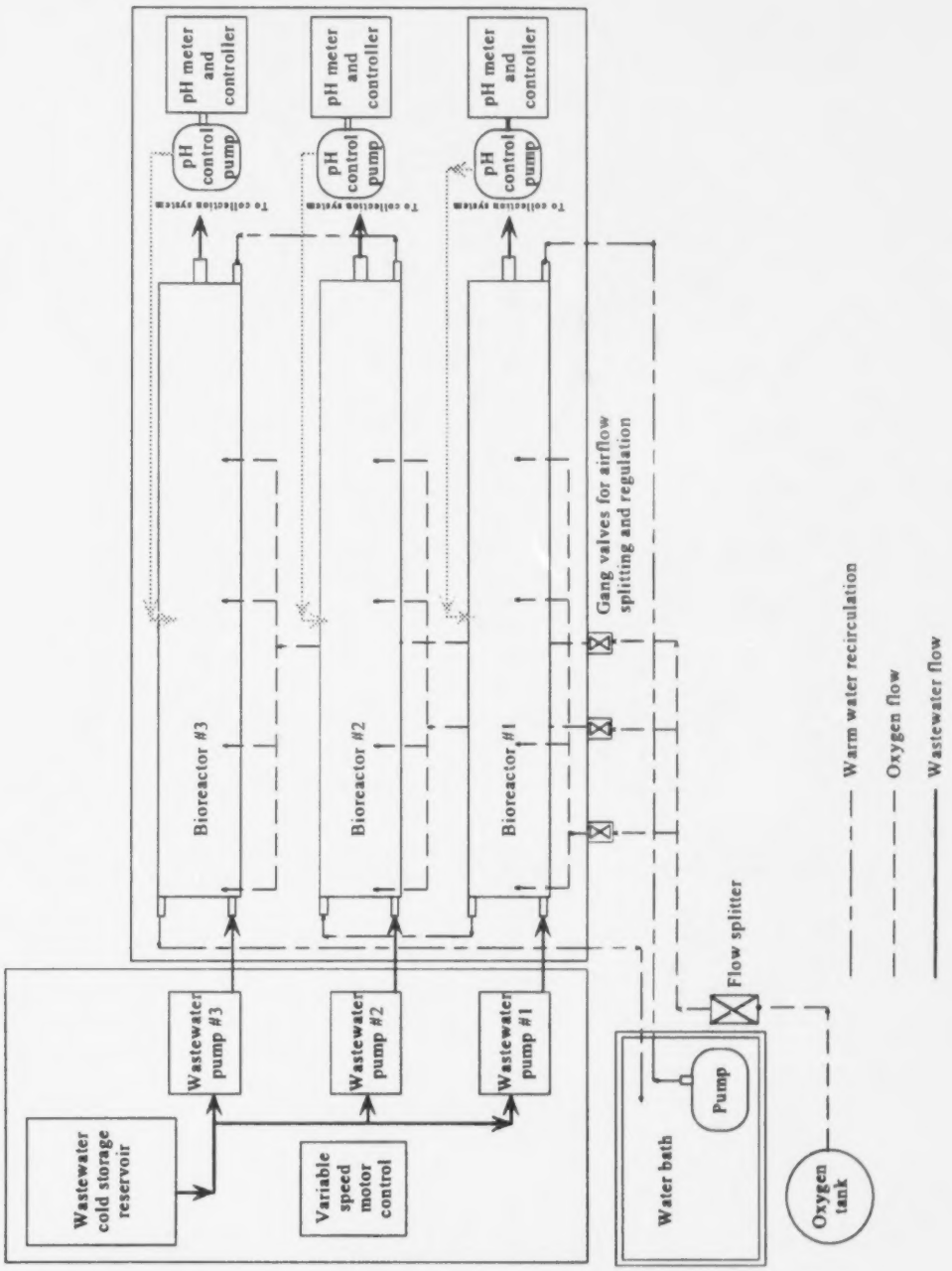


Figure 3.6. Schematic of the IF-RBC set up in the laboratory with all three reactors running. Oxygen flow, wastewater flow, and hot water recirculation are shown.

3.3.2 pH control system

In the bioreactors, pH was controlled by two pumps, one feeding acid and the other feeding base. The pumps were on automatic controllers that, based on the pH meter reading in cell 3 of the bioreactors, would switch one of the two pumps on if the pH fell outside the range of 4.0 to 5.0 pH units. Acid or base would feed into the beginning of the third bioreactor cell until the pH meter registered a change back into the acceptable range for fungal decolorization.

Pumps for pH control were two Buchler Mono-static pumps and one Cole-Parmer (Model 7553-80, 1 to 100 rpm) pump with a Masterflex speed control, depending on the number of reactors being run and the pH fluctuation during each run. pH was monitored and controlled with three Fisher Accumet® pH meters (models 805 MP and 915) and with three Fisher Scientific AC load interface controllers.

3.3.3 Polyurethane foam

Sheets of 35.6 and 17.8 mm thick Foamex polyether foam were used as an immobilization material for the fungal biomass. The polyether foam was selected because it was much more durable under the conditions set for the bioreactor (low pH, moderate temperature) than a polyester foam manufactured by the same company. A pore size of 80 pores/100 mm or 20 pores-per-linear-inch (ppi) was used for the foam in the bioreactor and batch tests. For waste treatment, a foam density of 32 kg/m^3 (2 lb/ft^3) was recommended by the manufacturer. Product specifications relating to foam lifetime and chemical stability are shown in Table 3.4.

Discs were cut for the bioreactor from the sheets of foam. A cutting guide was used to cut discs 100 mm in diameter from the foam. For the bioreactor, 16 of the 35.6 mm thick discs or 32 of the 17.8 mm discs were required.

Table 3.4. Foam lifetime and chemical stability (Foamex 1995)

	Polyester	Polyether
Water		
Room temperature	> 5 years	Probably > 10 years
71 °C	8 weeks	Probably > 1 years
93 °C	9 days	Probably > 6 months
Acids		
HCl - concentrated	Dissolves in 2 minutes	40 days
- dilute	10 days	> 2 years
H_2SO_4 - concentrated	Dissolves in 5 minutes	Dissolves in 5 minutes
- dilute	40 days	> 2 years
HNO_3 - concentrated	Dissolves in 25 minutes	Dissolves in 3 hours
- dilute	10 days	> 2 years
Bases		
NaOH - 36%	2 days	1.5 to 2 years
- dilute	12 days	> 2 years
NH_4OH - concentrated	12 days	> 1.5 years
- dilute	4 months	> 2 years
Solvents		
Hydrocarbons	Slight swelling (5 to 10%)	Moderate swelling (20 to 30%)
Chlorinated	Severe swelling (50 to 75%)	Severe swelling (50 to 100%)
Alcohols	Slight swelling (5 to 10%)	Moderate swelling (30 to 50%)
DMF or DMSO (hot)	Dissolves	Dissolves

Notes: Salts such as NaCl have no additional adverse effect. Salts that have a pH to acid side will accelerate rate of hydrolysis (degradation) to a greater extent than salts that have a basic pH. Dilute is considered to be 1 Normal concentration.

3.3.4 Fungus growth under continuous flow conditions

Several methods of starting the fungal cultures on the foam supports were used throughout this research. Both in-situ and external inoculation were tried to examine which method produced the best results.

For the in-situ cultures, a spore suspension was made by scraping spores from the cereal agar plates into sterile nutrient solution under aseptic conditions. Anywhere from 8 to 15 plates per reactor were used to initially inoculate the reactors, with more spore plates added as necessary. Approximately 0.1 mL Tween 80 per 100 mL solution was added to the spore suspension before mixing. After the reactor and foam discs on the shaft were washed and autoclaved, the reactors were filled with solution and the spore suspension was poured over the sponges. Some initial runs did not use a spore suspension; rather the spores were added directly to the liquid solution in the reactor. The sponges were set to rotate at a low speed, less than 2 revolution per minute.

Two methods were used during the in-situ growth to provide a constant supply of nutrient solution to the fungus. In one case, recirculation of the solution was used so that fresh solution would be pumped into the influent port, travel through the reactor, exit the effluent port and be collected in a closed container for return to the reactor. This technique allowed approximately two times the reactor volume of nutrient solution to be available for the fungal growth. Fresh media or sterile water was added daily to replace evaporation losses. At the midway point of the growth period, the media that had undergone many recycles was replaced with completely fresh solution in the recirculation container. In the second method, no recirculation was used. As the batch studies indicated, fresh solution was not necessary during the growth period because the nutrient solution contained sufficient quantities of glucose and minerals to allow adequate growth before decolorization. The reactors were filled and inoculated with fungal spores with no flow through the system. Fresh solution or sterile deionized water was used to replace evaporation losses.

In the external method of starting the reactors, the foam discs were autoclaved in large beakers. The spore suspension and sufficient nutrient solution to completely immerse the sponges were aseptically added to the beakers. The beakers were incubated at 37 °C for 4 to 7 days, with the sponges flipped over ever second day. After sufficient mycelial growth was attached to the sponges, they were transferred to the bioreactor shafts and placed in the bioreactor.

After each growth or regeneration period, the reactors were drained of nutrient solution with a pump and filled with the wastewater to be decolorized. This helped to reduce the number of hydraulic retention times necessary before the effluent sample was representative of fungal decolorization rather than dilution by the colourless solution.

3.3.5 Effluent collection system

After the wastewater flowed through the last bioreactor cell and into the overflow zone, it was collected by gravity drainage through the effluent port into a 500 mL vacuum flask. The vacuum flask was drained into a 4 L flask set on a lower level than the vacuum connection on the small flask. This design allowed fresh sample to collect in the small flask for sampling at specific times without having a composite sample over 24 hours, or whatever sampling interval was being used for the test. For example, with a 24 hour retention time, the 500 mL flask would represent the last 3 hours of bioreactor effluent. The effluent collection system is shown in Figure 3.7.

When the bioreactor was run in batch mode, samples were withdrawn with a 60 mL syringe from the last cell of the bioreactor. Fresh effluent was added to the first or second cell to replace the sample that was removed. To make up for evaporation losses, deionized water was added with the syringe until the liquid level reached a meniscus at the overflow partition.

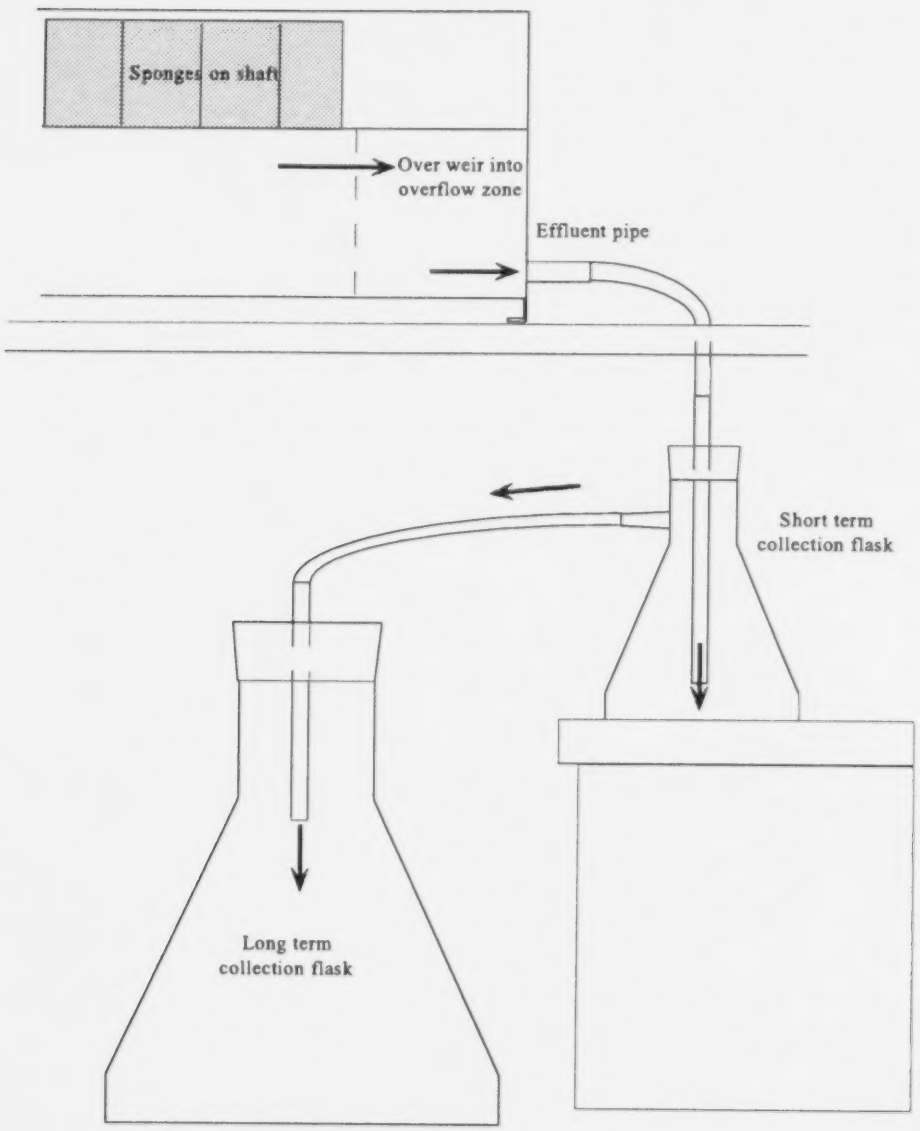


Figure 3.7. Effluent collection system for IF-RBC in continuous flow operation

3.4 Batch test studies

3.4.1 Batch test studies - fungus growth and transfer

The fungi were cultured in oxygenated liquid nutrient solution for 7 days then transferred to the pulp mill effluent samples. The samples were aerated with 100% oxygen and incubated at 37°C for 8 days, with colour and pH determinations daily. After the first test where the biomass was suspended in the liquid effluent, the remaining tests used sponge cubes as support for the fungal biomass to better simulate the IF-RBC conditions. Either a spore suspension (Kirk *et al.* 1978) or spore plate scraping was used for the culture innoculum to add roughly 9×10^8 spores to 200 mL of nutrient solution prepared as previously discussed. Tween 80 was added to all cultures under growth conditions to reduce the hydrophobic nature of the spores. Aseptic techniques were used in all cases.

Cultures were incubated at 37 °C in a water bath. Oxygen was bubbled through the liquid to maintain saturated oxygen conditions. Once the required growth period was completed (2 to 7 days, depending on the test), the immobilized fungus was transferred to the pulp mill wastewater to begin the decolorization test. After the liquid was drained from the Erlenmeyer flasks, the foam cubes were carefully removed from the flask and allowed to gravity drain before transfer to the warm wastewater. Controls, without fungus, were run with each set of batch tests. To simulate the slight dilution from the liquid remaining trapped in the sponges, sterile sponges were soaked in sterile deionized water then transferred to the wastewater using the same procedure as for the fungus inoculated sponges. The controls were aerated and incubated under the same conditions as the test samples.

Table 3.5 provides a summary of the batch test conditions for the suspended growth test and for the eight attached growth tests. All tests used ASB wastewater which had been ozonated at 80 mg/L over 30 minutes at room temperature. The three strains of fungi are identified by their culture collection number: UAMH 3642, UAMH 4521, and ATCC 24725.

Table 3.5. Summary of batch test conditions for batch tests run to October 1995

Test	Conditions
Suspended Growth #1	Effluent pH buffered to ~ 4.5 1% glucose added Vitamins and minerals added Wastewater heated at 60 °C for 30 minutes
Attached Growth #1	Effluent pH buffered to ~ 4.5 1% glucose added Vitamins and minerals added Wastewater heated at 60 °C for 30 minutes
Attached Growth #2	Repeat of Attached Growth Test #1
Attached Growth #3	Effluent pH buffered to ~ 4.5 1% glucose added Wastewater heated at 60 °C for 30 minutes
Attached Growth #4	Effluent pH buffered to ~ 4.5 1% glucose added No heat treatment of wastewater
Attached Growth #5	Effluent pH buffered to ~ 4.5 1% glucose added No heat treatment of wastewater
Attached Growth #6	Repeat of Attached Growth Test #5
Attached Growth #7	Testing varying concentrations of glucose with ATCC 24725 and UAMH 3642 strain in ozonated (80 mg/L) pulp mill effluent
Attached Growth #8	Testing varying concentrations of glucose with UAMH 4521 strain in ozonated (80 mg/L) pulp mill effluent

3.4.2 Buffer addition for pH control

A buffering system was chosen to give a pH of 4.5 ± 0.5 over 8 to 10 days with the biological system producing unknown concentrations of organic acids. As the buffer could not provide a carbon source for the fungus, some buffers, such as those containing acetic acid, were not considered. Potassium pthalate does not provide an easily assimilable carbon source for *Phanerochaete chrysosporium* (Kirk *et al.* 1978). Concentrations of 0.004 M NaOH and 0.025 M potassium pthalate were used to maintain the pH approximately within the optimum range for the fungus growth during the batch tests.

In all batch tests, both the ozonated and raw ASB wastewater was buffered to a pH of approximately 4.5 ± 0.5 with sodium hydroxide (0.004 M) and potassium phthalate (0.025 M). As the pH of the raw wastewater was slightly higher than the ozonated samples before buffering, a slight difference of about 0.5 pH units remained between the two samples during the tests.

3.4.3 Sampling methods

Colour samples were taken daily with a 60 mL syringe and a long needle, using care to avoid removing any mycelium. At least 30 mL of liquid was withdrawn for colour analyses, which followed the Canadian Pulp & Paper Association Standard H.5P (CPPA 1974) for colour determination of pulp mill effluents as described previously. In the batch tests, to prevent the quantity of fungus from becoming disproportionate to the amount of wastewater remaining after sampling, one foam cube, and the attached fungal growth, was removed from the flask and discarded each time a sample was taken.

3.4.4 Growth test methods

3.4.4.1 Determination of fungal dry weights

A growth rate test was conducted on strain ATCC 24725. Erlenmeyer flasks were sterilized with five foam cubes, approximately 18 to 20 mm on each side, in each. The dry weight of the cubes was recorded before 100 mL of nutrient solution was added to each flask. A spore suspension was made from 18 plates and 280 mL of nutrient solution blended with 0.25 mL of Tween 80 for 20 to 30 minutes at medium mixing speed until homogenous. 15 mL of the spore suspension was pipetted into each flask. The flasks were incubated in a water bath at 37°C and were aerated with pure oxygen.

Two flasks were filtered immediately through a 0.45 µm sterile, previously weighed filter. The filter and the sponges were oven dried at 103 °C for 24 hours and weighed again to find the initial weight of the spores added to the flasks. Every day after the first day, two flasks were removed and the filtering, drying, and weighing process repeated. The filter and sponges weights were subtracted from the total dry weight to give the dry weight of the fungal mycelium over time.

3.4.4.2 Effect of culture age on decolorization experimental setup

To determine the effect of culture age on fungal decolorization ability, another batch test was set up where the *Phanerochaete chrysosporium* was cultured as described previously for batch tests, then transferred to ozonated, glucose enriched pulp mill wastewater. The culture ages ranged from two to nine days old in increments of one day, with each day done in duplicate for the strain ATCC 24725. All foam cubes were left in the flask throughout the experiment. 30 mL colour samples were taken every second day for analysis. At the end of eight days for each set of duplicates, the samples were filtered

through a 0.45 µm filter, oven dried, and weighed to find the mass increase compared to both the initial and the previous growth test.

3.4.5 Nitrogen analysis

Pulp mill effluent samples with added NH_4Cl were analyzed for nitrogen as NH_3 by the University of Alberta Limnology Laboratory. The Indo-phenolhypochlorite method (Phenate method 417C) was used on a Technicon AutoAnalyzer (APHA 1989).

3.4.6 Glucose analysis

Glucose concentrations were determined by the University of Alberta Department of Laboratory Medicine and Pathology using the Hexokinase method on a Hitachi 911 glucose analyzer.

3.5 Colour change in ozonated ASB effluent samples tests

3.5.1 Experimental design

The first test was carried out in triplicate at three temperatures, 4 °C, 22 °C, and 40 °C, with pure oxygen bubbled through 1.8 L of ozonated (80 mg/L over 30 minutes) pulp mill wastewater contained in 2 L flasks with foam stoppers. Samples (30 mL) were withdrawn daily for colour analysis and every second day for BOD analysis. HPC's were done on composite samples on day 0, 4, and 10 to determine whether the microbial population increased. One control, with no samples removed, was also run at each temperature to determine evaporation losses.

The second run was a factorial design experiment with temperature and pH as the factors to be tested and colour, pH change, BOD, and HPC as the measured variables. Table 3.6 outlines the experimental conditions. In 2 L flasks aerated with pure oxygen, 1.4 L of ozonated (80 mg/L over 30 minutes) was used for each sample. Colour analyses required 30 mL of sample daily; 130 mL was removed on days 2, 5, and 10 for BOD analysis; HPC's were done on the remaining volumes from the BOD analyses on days 0, 5, and 10 by combining the duplicate samples to obtain a more representative indication of biological activity.

Table 3.6. Factorial design for changes in pulp mill wastewater characteristics after ozonation

	Low setting	Medium setting	High setting
Initial pH	4.0	5.6	7.2
Temperature	4 °C	22 °C	40 °C
Measured variables:	True colour	pH change	BOD	HPC

Duplicates at two levels, plus centre point triplicates were run in order to separate the main effects and interactions, as well as to evaluate the standard error in the measurements.

3.5.2 Experimental methods

Temperature was controlled at 4 °C by placing the flasks in a large cooler with the oxygen line running in through the opening of the sliding door. The two remaining temperatures, 22 °C and 40 °C were maintained with constant temperature water baths. True colour, pH, BOD, and HPC's were determined as previously described.

The initial pH was not adjusted for the first run. During the second run, the pH was brought to the necessary level by the addition of concentrated HCl or NaOH before the test was started. The original pH was selected to be the high level for the test, while the lower level was set at the lower boundary of the optimum range (4 to 5 pH units) for fungal decolorization.

3.6 Toxicity tests

3.6.1 Steel toxicity tests

Several batch test experiments were run to identify possible materials causing toxicity or decolorization inhibition with the *Phanerochaete* in pulp mill effluent. Because a direct cause for decolorization inhibition could not be determined without trial and error testing, the steel, the welding rods, and the welded steel were all evaluated in batch tests. One week old plates of ATCC 24725 fungus were scraped into standard nutrient solution to make a mycelium suspension with the addition of 0.1 mL of Tween 80 per 100 mL of nutrient solution. For each individual flask, half a plate of mycelium growth was added through addition of the suspension in the correct proportion to 200 mL of nutrient solution.

The flasks, containing five foam sponges each, were incubated in a water bath at 37 °C for 4 days. The liquid was then drained and the fungus was transferred to 250 mL of ozonated (80 mg/L over 30 minutes) pulp mill effluent with 1% glucose as a carbon source. All other parameters, such as pH, temperature, and oxygenation were the same as in previous tests. As no buffer was added in order to better simulate the bioreactor conditions, the sample pH was measured every second day and base or acid was added as necessary to maintain the optimum pH range. Samples were taken every second day for colour analyses. The test setup is presented in Table 3.7.

Table 3.7 - Test setup for batch test toxicity evaluation of stainless steel, welding rods, and welded stainless steel

Test and Sample	Growth conditions	Decolorization conditions
Test #1 - 1A and 1B	Regular conditions	Regular conditions
Test #1 - 2A and 2B	Steel plates in growth	Steel plates in effluent
Test #1 - 3A and 3B	Steel plates in growth	Regular conditions
Test #1 - 4A and 4B	Regular conditions	Steel plates in effluent
Test #2 - 1A and 1B	Regular conditions	Regular conditions
Test #2 - 2A and 2B	Welding rod pieces in growth	Welding rod pieces in effluent
Test #2 - 3A and 3B	Welded steel plates in growth	Welded steel plates in effluent
Test #3 - 1A, 1B, and 1C	Regular conditions	Regular conditions
Test #3 - 2A, 2B, and 2C	Welded steel plates in growth	Welded steel plates in effluent

In Test #1, 25 mm x 50 mm plates were cut from 304 grade, 2B finish stainless steel. All plates had a hole punched into the top center of the long side of the plate. One third of the plates were heated to red hot with an acetylene torch to simulate successive autoclaving and to remove the shiny finish. These plates were then soaked overnight in deionized water that had the pH adjusted to approximately 2 with HCl. The plates were autoclaved before three were suspended in each 500 mL Erlenmeyer flasks by plastic line so that the bottom edge rested on the bottom of the flask and the majority of the surface area was exposed to pulp mill effluent liquid. All samples were done in triplicate.

In Test #2, a 304 and a 318 grade steel welding rod were cut into approximately 50 mm pieces, autoclaved, and aseptically placed into the Erlenmeyers. Twelve pieces were added to each flask. These rods were transferred from the growth flask to the decolorization test flask along with the fungus attached to the foam cubes. Welding lines were added to some of the steel plates after the completion of the first test to evaluate the combined effect of welding and the steel plates. One line of welding was done across one side of the steel plate using a 304 grade steel rod. These plates were autoclaved and aseptically suspended in the flasks as described above. The nutrient solution liquid was drained from the flasks, the sponges were removed to drain, wastewater was added to the flask, and then sponges were finally returned to the flasks for testing. All samples were done in duplicate.

Test #3 was a repeat of test #2, except in triplicate to verify results. When the test did not show the same results, a fourth trial was conducted with corrosion removed from the welded steel plates by sanding.

3.6.2 Metal analysis

Metal concentrations analyses were contracted out to Enviro Test Laboratories, Edmonton Alberta for the nutrient solution analysis and to University of Alberta Spectral Services, Chemistry Department, Edmonton Alberta. Both metal concentrations were determined with Inductively Coupled Plasma (ICP) Spectrophotometry using metal solutions as standards and following APHA 3120B/3030F (APHA 1989). Samples were preserved with nitric acid to below pH 2.0 and either filtered through a 0.45 µm filter for dissolved metals analysis or not filtered for extractable metals.

3.6.3 Metal toxicity tests

After metal concentrations were determined in pulp mill effluent incubated with steel plates with and without fungus present, the metals detected were added to pulp mill effluent with fungus present to evaluate individual and combined toxicity. Test setup for fungus cultures was as described in the steel plates additon tests.

Copper, chromium, iron, and nickel solutions were prepared from sulphate salts of each metal according to the concentrations detected in the metal analysis. All metals except for chromium were dissolved in deionized water. Nitric acid was added to the chromium solution to aid in dissolution. Standards were made up in 125 mg/L for chromium, 30

mg/L for copper, 2625 mg/L for iron, and 500 mg/L for nickel. These were pipetted in the appropriate volumes (0.5 to 2.5 mL) to obtain 0.5 mg/L chromium, 0.06 mg/L copper, 10.5 mg/L iron, and 2.0 mg/L nickel.

The first test used metals added only in the decolorization stage. Fungus was pregrown in nutrient solution then transferred to 250 mL of ozonated ASB pulp mill effluent with metals added individually and all together. Each metal and the control without metals present were set up in duplicate tests. Samples were taken every second day for six days for colour analysis.

The second test used metals added both in growth and decolorization. Identical metal concentrations were used in both stages. Samples were again run in duplicate. Controls were also set up to determine colour increase from the metals present in solution. 10.5 mg/L iron was added to pulp mill effluent and aerated at 37 °C without fungus present. The same method was used to find colour increase without metals and with all four metals added to the ozonated pulp mill effluent.

3.7 Improved medium evaluation

Following procedures outlined in Mao (1996), batch tests were conducted to evaluate decolorization with the improved medium. ATCC 24725 plates of seven day old mycelium were scraped into 300 mL of the basic nutrient solution in cotton stoppered 500 mL Erlenmeyer flasks and incubated at 37 °C for 4 to 5 days without agitation and under fully oxygenated conditions. The liquid was drained from the fungal biomass through a filter in a Buchner funnel under aseptic conditions. After gravity draining the nutrient solution from the fungal biomass, the biomass was washed with sterile 0.8% NaCl solution to remove any remaining nutrient solution. After draining again, the fungus was weighed and proportioned in the desired amounts for the tests.

In testing the improved medium, 150 mL of raw or ozonated ASB effluent samples were added to 250 mL Erlenmeyer flasks with cotton and/or foam stoppers at the top. The wastewater had been previously mixed with the minerals and nutrients to be tested and had the pH adjusted to 4.5 units.

To repeat the previous work (Mao 1996), 10.0±0.5 g of fungus (wet weight) were added to the wastewater and incubated at 37 °C for the duration of the test. Oxygen was added

through a 1 mL disposable pipette extending into the liquid. Colour analysis was done on 10 mL samples that had been withdrawn every second day using a syringe with a long steel needle. Samples were run in duplicate unless otherwise noted.

3.7.1 Factorial experiment for evaluation of improved medium

The effect of improved medium was also evaluated in two factorial tests, one using ozonated effluent and the other using raw effluent. Both were designed to examine the impacts of varying concentrations of three parameters, Tween 80 addition, $MnSO_4$ concentration, and level of buffering, and to determine which variable had the greatest significance to colour removal without an additional carbon source. These three variables were selected for study because they were the only factors that differed from the initial batch tests where colour removal did not occur without glucose present. A 2^3 factorial experiment was designed to evaluate which factor or interaction of factors was important in improving the decolorization without additional glucose. The test was run as a batch test, in duplicate, with center point triplicates. Tables 3.8 and 3.9 show the test setup for the factorial experiments.

Table 3.8. Concentrations of Tween 80, buffer, and $MnSO_4$ used in the factorial experiment evaluating improved medium

	Low level (-)	Medium level (0)	High level (+)
Tween 80	0.10 mL/L	0.50 mL/L	0.90 mL/L
Buffer	0 mM NaOH	4 mM NaOH	8 mM NaOH
	0 mM KHP	25 mM KHP	50 mM KHP
$MnSO_4 \cdot H_2O$	0.1 mM	0.5 mM	0.9 mM

Table 3.9. Factorial design for evaluation of improved medium

Run	Sample ID	Tween	Buffer	$MnSO_4$
1	A1, A2	low	low	low
2	B1, B2	high	low	low
3	C1, C2	low	high	low
4	D1, D2	high	high	low
5	E1, E2	low	low	high
6	F1, F2	high	low	high
7	G1, G2	low	high	high
8	H1, H2	high	high	high
9	I1, I2, I3	medium	medium	medium

The fungal biomass was cultured as previously described for the improved medium tests. In this set of tests, 150 mL of ozonated (80 mg/L) wastewater with the appropriate concentrations of the three factors being tested as well as the same quantities of the other minerals in the improved medium was used. Because of limited fungal biomass growth, 5.7±0.3 g wet weight of fungus were added to each flask instead of the regular 10 g. Test flasks were incubated and samples taken as for the improved medium test.

The factorial experiment was also done on the raw ASB wastewater using the same setup. Instead of a weighted mass of fungus as used in the ozonated wastewater test, flasks with foam cubes and a set innoculum similar to the steel toxicity test were used. Half a plate of one week old fungus was used for each flask.

4. RESULTS

The transition phase of this project, formerly termed "Advanced engineering system for decolorization and dechlorination of pulp mill effluent", was intended to focus on optimization of the immobilized fungi rotating biological contactor. After it was proposed in October 1994, work began with the existing bioreactor (Plexiglas) and then newly designed reactors (stainless steel). From January to June 1995, the work focused on bioreactor performance. Batch tests began in June after continually poor results from the bioreactor runs. These batch tests were designed to test on a small scale a variety of conditions which could then be applied to the bench scale bioreactors. Table 4.1 outlines the work completed from January 1995 to June 1996 on the combined ozonation/biological process for decolorization of pulp mill effluent.

Despite no colour reduction in the bioreactors, full parameter testing including AOX, TOC, BOD, COD, and microbiological counts, as well as colour were being run on bioreactor effluent. After the new bioreactors were setup, colour became the main research focus on the assumption that colour reduction would be indicative of changes in the other parameters. TOC was measured in the bioreactor effluent until an estimate of the number of hydraulic retention times (HRTs) necessary to clear the reactors of nutrient rich growth solution was obtained.

In the batch tests, colour was again the primary focus when different conditions were tested and variables eliminated as a search began to narrow down what caused the lack of bioreactor colour reduction. Research from the earlier phase of this project (Mao 1996) was repeated and used to design further experiments to test the hypotheses developed in that research as well as other research presented in the literature. Some basic investigations, well documented in literature, were replicated to rule out factors that had potential to cause laboratory specific results. When the batch tests yielded positive information, this was applied to the bioreactors with no success. Further batch tests continued to provide more information on possible inhibitors to the process.

Table 4.1. Summary of work completed during transition phase, January 1995 to June 1996

Date	Work Completed
January 1995	33 TOC, 33 BOD, 21 AOX, 49 COD, 55 colour, 5 HPC, 5 mold New ozonation system set up
February 1995	Attempt to control possible competing organisms with varying concentrations of ozone and with an in-line heater at 50° to 60°C 36 TOC, 36 BOD, 32 AOX, 34 COD, 54 colour, 9 HPC, 7 molds, 7 yeasts
March 1995	18 TOC, 19 COD, 10 AOX, 20 colour, 6 HPC, 6 molds, 6 yeasts
April 1995	13 TOC, 14 COD, 14 AOX, 28 colour, 11 BOD, 4 HPC, 4 molds, 4 yeasts New bioreactor constructed and put into operation. Hydraulic dye tests conducted to determine flow characteristics. Effluent heat treated at 60°C for 30 minutes to reduce initial microbial populations
May 1995	22 TOC, 11 COD, 8 AOX, 60 colour, 14 BOD Two new bioreactors of same design added to setup for running parallel tests Nutrient solution analysis completed
June 1995	60 TOC, 5 COD, 180 colour, 5 BOD New samples of *Phanerochaete chrysosporium* obtained from the Devonian Botanical Gardens and the American Type Culture collection. Batch tests started - Suspended Growth Batch Test #1
July 1995	294 colour tests Comparison of three strains of *P. chrysosporium* with and without nutrients present in raw and ozonated wastewater. Attached Growth Batch Tests #1 and #2
August 1995	34 NH_3 tests, 228 colour and pH tests Batch tests conducted to determine necessity of vitamin and mineral addition and of heat treating wastewater before fungal treatment Attached Growth Batch Tests #3 and #4
September 1995	292 colour and pH tests, 9 BOD Batch tests run to reduce variability in results and to determine if heat treating is necessary for colour reduction Attached Growth Batch Tests #5 and #6
October 1995	22 AOX, 250 colour and pH tests, 75 glucose concentration, 52 BOD, protein analyses Batch tests conducted to find effects of varying glucose concentrations Attached Growth Batch Test #7 and #8
November 1995	246 colour and pH, 161 BOD tests Batch tests to find colour change after ozonation Ozonation Colour Change Tests #1 and #2 Restarted Bioreactor #1 with UAMH 4521 and ozonated effluent with 0.4% glucose and thiamine at 72 hour HRT Restarted bioreactor #2 with ATCC 24725 with old inoculation method

Table 4.1 continued. Summary of work completed during transition phase, January 1995 to June 1996

Date	Work Completed
December 1995	120 colour and pH tests, 23 BOD tests Ran bioreactors on 1% glucose, HRT 72 hours
January 1996	58 colour and pH tests, 7 BOD tests, 2 AOX and TOC tests Effect of culture time on decolorization tests run Growth rate test based on mycelial dry weight changes Bioreactor #1 and #2 started as batch reactors, #1 with 1% glucose, #2 with no glucose
February 1996	128 colour and pH tests, 3 BOD tests, 2 AOX and TOC tests Growth rate test run for 21 days measuring mycelial dry weight Bioreactor batch runs continued with no sponge rotation and 1% glucose addition Concentrated raw and ozonated samples used in testing
March 1996	107 colour and pH tests Improved medium tests run Effect of stainless steel on fungus decolorization evaluated Bioreactors continued to be run with no sponge rotation, 1% glucose and no flow
April 1996	116 colour and pH tests, 5 BOD tests Improved medium evaluation continued with further batch tests including factorial evaluation using ozonated effluent Effect of stainless steel on fungus decolorization continued with welded steel coupons
May 1996	122 colour and pH tests Factorial evaluation of improved medium continued with ozonated effluent Effect of stainless steel on decolorization continued Metals analysis run on ozonated effluent samples aerated with welded steel coupons suspended in the effluent, both with and without fungus present
June 1996	158 colour and pH tests, 16 BOD tests Factorial evaluation of improved medium using raw wastewater batch tests Biodegradability of improved medium assessed Possible adsorption of colour evaluated in fungus with improved medium added Effect of metals found in May analysis tested individually and all together

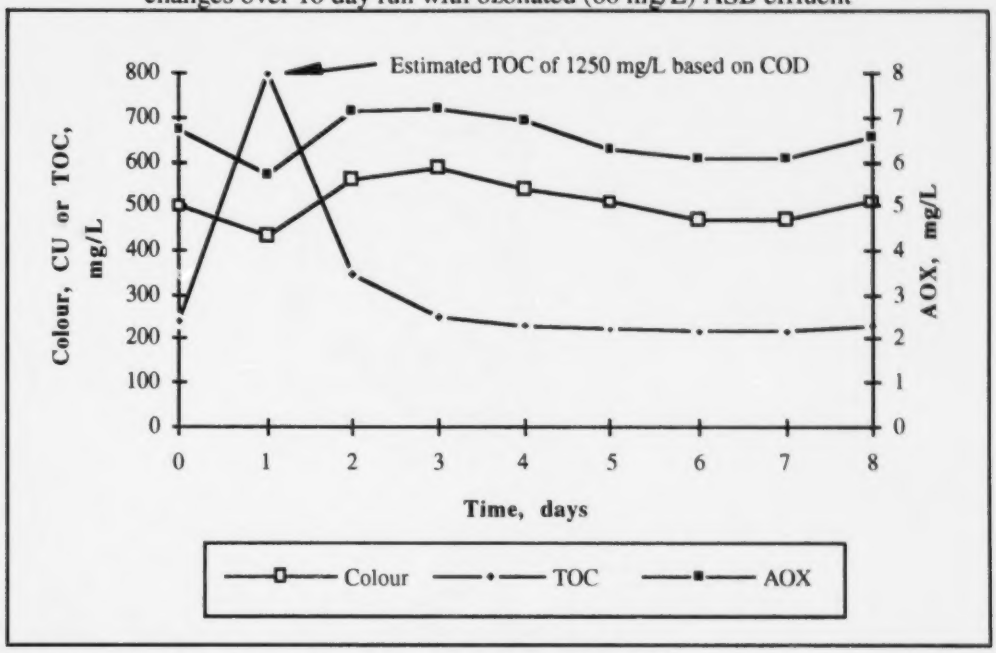
4.1 Immobilized Fungi Rotating Biological Contactor results

The original Plexiglas bioreactor used in previous studies (Mao 1996) was run under varying conditions from January to April 1994. Evaporation (greater than 20% of the reactor volume in 24 hours) proved to be excessive and make-up liquid (either ozonated effluent or deionized water) had to be added. A minimum of 48 hours, and possibly up to 72 hours, was required to stabilize the bioreactor after ozonated ASB effluent addition at a HRT of 24 hours. Effluent samples taken before this time still contained nutrient solution which increased the TOC and decreased the colour because the samples were diluted with the colourless high carbon solution. Short circuiting with areas of no flow was a problem with the bioreactor.

Ozonated ASB effluent with the ozone dose ranging from 40 mg/L to 140 mg/L was run through the bioreactor and effluent quality measured. No biological colour reduction was found at any of the ozone concentrations. Figure 4.1 shows data from a typical run for this bioreactor. Dilution caused the initial colour reduction as the high spike of TOC from the nutrient solution shows on the graph. Colour and AOX show the same pattern where an initial decrease from dilution occurs, followed by increasing values to the end of the run. When the TOC reached a constant value, all growth solution had been removed from the reactor. The colour increased until the run was halted and the reactor was again filled with nutrient solution in attempts to regenerate the fungus. Bioreactor results for all parameters tested from January 1995 to June 1996 are given in Appendix B. No colour reduction was found with the Plexiglas bioreactor.

High turbidity noted in the effluent was ascribed to fungal sloughing in addition to bacteria and yeast contamination. Microbiological work, including HPC's, presumtive yeasts, and molds/fungi counts, determined that a mixed population of microorganisms were present in the bioreactor and that the source of the "contaminants" was mainly the ASB effluent added to the reactor. Lack of colour reduction was attributed to microorganisms other than *P. chrysosporium* competing for the limited biodegradable substrates. Ozonation was not effective at reducing the microbial population of the ASB effluent. Heat treatment was used to greatly reduce the microorganisms present before the ozonated effluent was added to the reactor. Table 4.2 shows the results of heating the ozonated effluent at 60°C for 20 minutes.

Figure 4.1. Typical bioreactor (Plexiglas) performance in terms of colour, TOC and AOX changes over 10 day run with ozonated (80 mg/L) ASB effluent



Graph based on data from March 13, 1995 to March 21, 1995. Colour was measured at pH 7.6 after the sample was centrifuged to remove solids.

Table 4.2. Effect of ozone dose and heat on microbial population in ASB effluent

	Unheated ASB effluent		Heated ASB effluent (60°C for 20 min.)	
Ozone Dose, mg/L	HPC (35°C, 48 hr), cfu/mL	Presumptive yeasts (20°C, 7 day), cfu/mL	HPC (35°C, 48 hr), cfu/mL	Presumptive yeasts (20°C, 7 day), cfu/mL
80	3.8×10^5	1.2×10^6	6	4.1×10^2
109	1.6×10^5	1.5×10^4	7	2.2×10^2
114	1.2×10^5	1.7×10^3	7	1.7×10^2

Microbial populations were reduced five logs in the case of HPC bacteria, and one to four logs in presumptive yeasts. Molds, not shown in the table, also decreased in concentration, but these fungi were not present in high numbers in the original sample, making measurement difficult. Comparing the effectiveness of ozone and heat treatment separately and in combination, ozone dose had little impact on bacterial populations in the ASB effluent while heat treatment was very effective at reducing the numbers. With

yeasts, the opposite trend was apparent. Increasing ozone concentrations decreased the presumptive yeast populations by two logs with an increase of 34 mg/L ozone. Although heat treatment reduced yeast populations in all samples, the final populations were similar regardless of the initial concentration after ozonation. This might indicate that a segment of the yeast population was resistant to both ozone and heat, surviving both treatments.

Beginning in April 1995, the bioreactors were run under various operating conditions without achieving colour reduction. Table 4.3 outlines the different experimental parameters that were altered during the test runs and the range on each of the conditions.

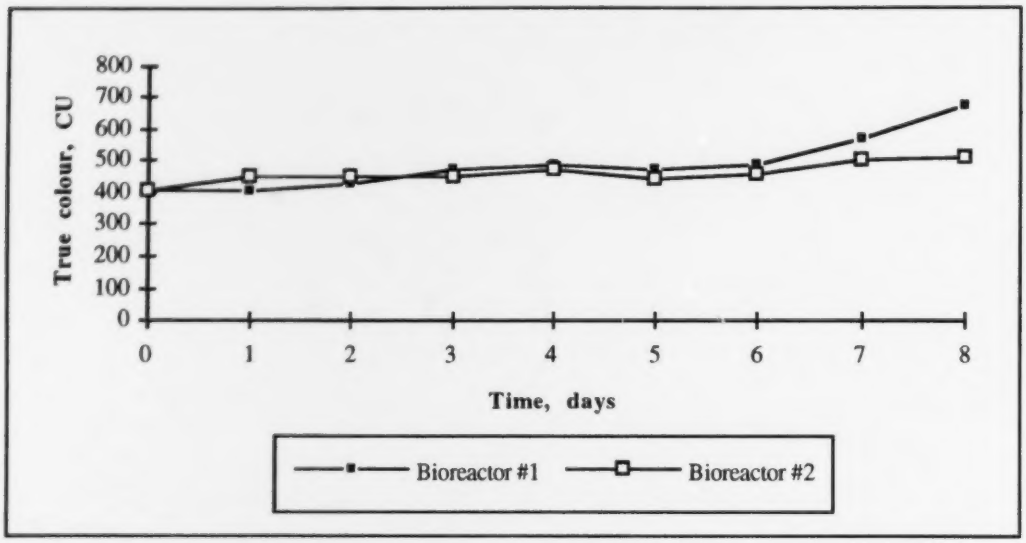
Figure 4.2 presents a typical run from two of the stainless steel bioreactors. Both were run with ozonated ASB effluent supplemented with 1% glucose. As this test was made to approximate the conditions in the batch tests, the sponges were not rotated and no wastewater was pumped through the bioreactors. Even under these conditions, no colour reduction was seen after 8 days and in bioreactor #1, the colour began to increase sharply after 6 days. Approximately 30 to 50 mL of deionized water was added daily to compensate for evaporation losses, but these losses were not significant enough to cause solute buildup which could inhibit the decolorization process. The fungus (ATCC 24725) was present in the bioreactor as both swabs and visible evidence showed. When the sponges were rotated at the end of the stationary period, a thick layer of fungal biomass was present on the outside of the sponges, and presumably into the sponge matrix also.

Table 4.3. Experimental parameters varied during bioreactor runs and the range tested

Condition or parameter	Range tested
Temperature	Constant at 35 to 37°C
pH	Controlled at 4.5 ± 0.5 pH units
Aeration	Extra dry oxygen bubbled through to saturation of liquid
Wastewater	ASB effluent ozonated at 80 mg/L over 30 minutes
System operation	Batch and continuous flow
Nutrient addition	0 g/L to 10 g/L glucose, Tween during spore addition
Culture method	In-situ and outside reactor
Sponge rotation	Stationary to 2 rpm
Growth period before effluent addition	2 to 7 days
Hydraulic retention time	12 to 72 hours
Heat treatment of wastewater	None, inline heater, or 60°C for 30 minutes
Phanerochaete chrysosporium strain	UAMH 3642, ATCC 24725, and UAMH 4521

Similar results were obtained for the stainless steel bioreactors regardless of the conditions used. All of the parameter ranges given in Table 4.3 were tested in several trials without successful colour removals. With all the necessary conditions needed for growth and decolorization by *P. chrysosporium*, the lack of colour removal was puzzling. Before batch tests were begun, microbial competition still seemed like a possible explanation for the bioreactor results. Even when conditions favorable to decolorization had been determined through small scale batch tests, these conditions applied to the bioreactors did not yield the same results. Further work was then continued to narrow down possible inhibitory factors in the decolorization process.

Figure 4.2. Typical results of bioreactor tests with stainless steel bioreactors run with ozonated (80 mg/L) ASB effluent and 10 g/L glucose added.



Data from bioreactors run March 12 to 19, 1996 with ATCC 24725 culture, batch mode and no sponge rotation.

4.2 Batch test results

Batch tests were conducted starting in June 1995 as a means of quickly evaluating decolorization performance on a small scale under a variety of conditions. Table 4.4 presents the underlying questions that drove the experiments and the conditions examined during the batch tests.

4.2.1 Carbon cosubstrate and nutrient requirements

In batch fungal systems, colour increased in most ozonated samples and raw ASB wastewater showed little colour change when glucose was not added to the wastewater. With glucose present, decolorization occurred in both raw and ozonated ASB effluent where greater than 50% of the initial colour could be removed by all three strains of *P. chrysosporium* after 8 days. One strain, UAMH 3642 showed some ability to remove colour in ozonated, but not raw, ASB effluent without a glucose source. UAMH 3642 achieved approximately 30% colour removals on average without glucose and over 40% with glucose addition. The other two strains, ATCC 24725 and UAMH 4521, both

increased the final colour or achieved less than 10% colour reduction which is within the experimental error. Figures 4.3 to 4.5 show the results of these initial batch tests. These results were the similar in both suspended growth and repeated attached growth tests, except that UAMH 3642 did not show significant colour reduction without glucose in the suspended growth test.

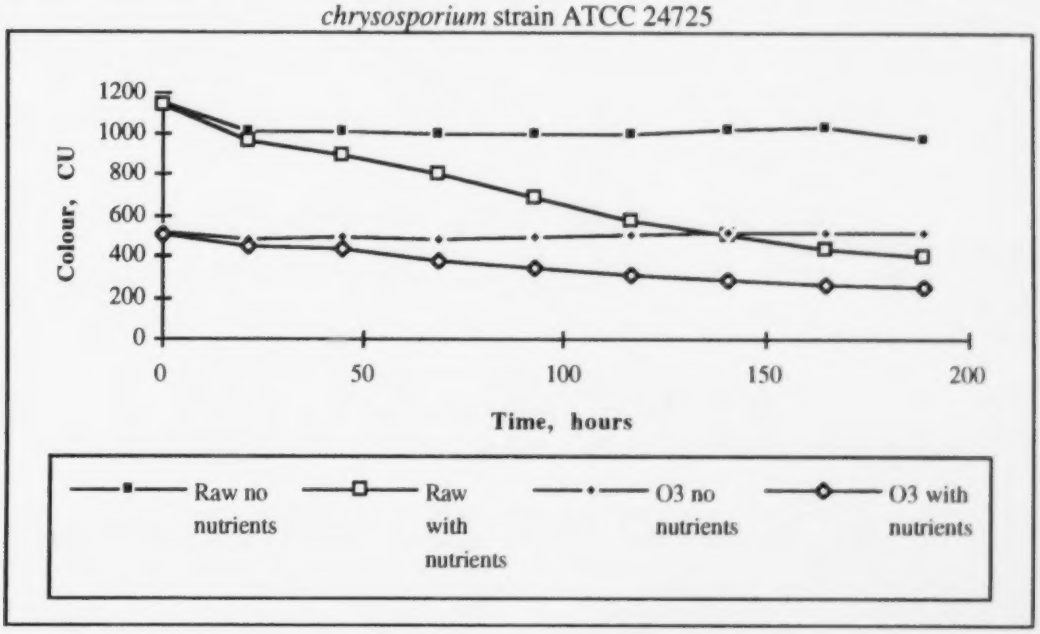
All samples were buffered with potassium hydrogen phthalate and sodium hydroxide. The phthalate did not serve as a readily available carbon source for *P. chrysosporium* in the raw and ozonated wastewater, as the lack of colour removal in samples without glucose shows. UAMH 3642 most likely did not use phthalate to reduce colour in the ozonated sample without glucose because similar reductions would then be expected in the raw sample if the phthalate was biodegradable to the fungus.

Table 4.4. Conditions evaluated during batch tests and means of evaluation

Condition	Means of evaluation
Is an extra carbon source necessary for decolorization by *P. chrysosporium*?	Glucose addition at 0 g/L and 10 g/L
Does ozonation improve decolorization by *P. chrysosporium*?	Comparison of colour removal in raw and ozonated wastewaters
Do vitamins and minerals improve decolorization?	Vitamins and minerals added in same concentration as nutrient growth solution compared to no addition
What is the minimum concentration of glucose necessary for decolorization?	Glucose addition at 0, 2, 4, 6, and 10 g/L
Is heat treatment of wastewater necessary to destroy competing microorganisms?	Heat treatment of ozonated effluent for 30 minutes at 60°C vs. no heat treatment
Which strain of *P. chrysosporium* performs the best in terms of decolorization?	Comparison of UAMH 3642, UAMH 4521, and ATCC 24725
Does glucose consumption differ in each strain and in relation to colour removal?	Glucose consumption measured in each strain
Does inoculation method affect growth and variability?	Spore suspension compared to adding a certain number of plates to each trial
Does an optimum growth period before decolorization exist?	Cultures of different ages transferred to wastewater
Does initial colour affect removal rate?	Concentrated ASB effluent used in decolorization experiments
Does the reactor material inhibit decolorization?	Steel coupons, welding rod pieces, and welded steel coupons added to batch tests
Which metal causes the most inhibition of fungal colour removal?	Iron, nickel, zinc, and copper suspected inhibitors. Wastewater spiked with these metals individually and in combination to test decolorization when present
Is colour reduction possible in "improved medium" wastewater without glucose?	Improved medium added to ozonated and raw ASB wastewater with fungus
What factor in the "improved medium" promotes colour reduction?	Factorial evaluation and BOD tests run on medium samples

Attached growth tests achieved better overall colour removals than suspended growth where the fungus did not have foam cubes present to provide an attachment surface. On average, 10% better colour removals with less fungus present were achieved in the attached growth tests over the suspended biomass experiment. Further testing usually involved foam cubes except when previously established methods were followed in replicating decolorization experiments.

Figure 4.3. Colour change in raw and ozonated (80 mg/L) pulp mill effluent with and without nutrient addition (10 g/L glucose and trace vitamins and minerals) for *P. chrysosporium* strain ATCC 24725



Abbreviations used in Figures 4.3 to 4.5 are as follows:

Raw: Unozonated ASB effluent

O3: ASB effluent ozonated with 80 mg/L over 30 minutes

No nutrients: Wastewater was buffered, but no glucose, vitamins, or minerals were added

With nutrients: Wastewater contains buffer, 1% glucose, vitamins, and minerals

All results are from attached growth test #2.

Colour is true colour measured at pH 7.6 and absorbance of 465 nm.

Figure 4.4. Colour change in raw and ozonated (80 mg/L) pulp mill effluent with and without nutrient addition (10 g/L glucose and trace vitamins and minerals) for *P. chrysosporium* strain UAMH 4521

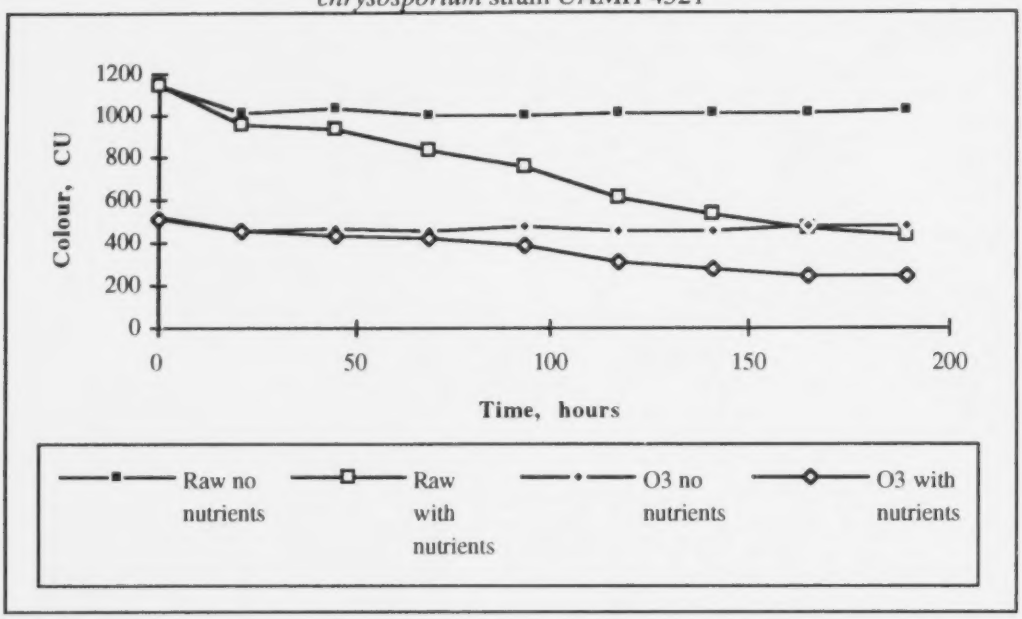
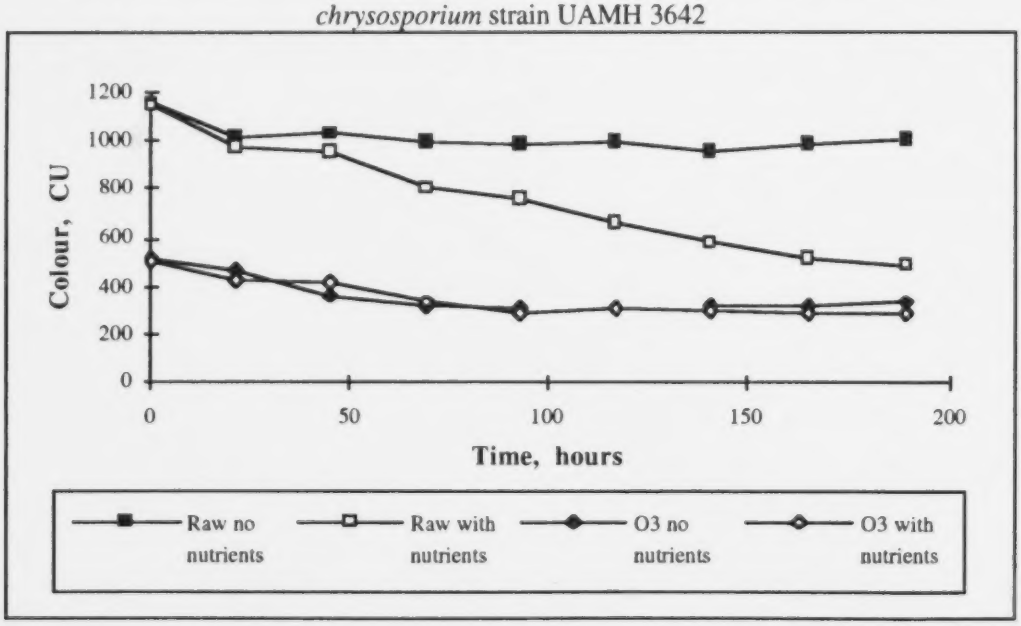


Figure 4.5. Colour change in raw and ozonated (80 mg/L) pulp mill effluent with and without nutrient addition (10 g/L glucose and trace vitamins and minerals) for *P. chrysosporium* strain UAMH 3642



In attached growth test #3, no vitamins or minerals were added to the wastewater to see if decolorization could proceed with only glucose and not these trace nutrients present. Although ATCC 24725 failed to decrease colour substantially (highest colour reduction was 17%) in any of the samples, UAMH 3642 and UAMH 4521 removed 47% and 48% of the colour in the ozonated samples and 59% and 45% in the raw samples, respectively on average after 7 days. The fungus did not remove colour in any of the samples in the next attached growth test, possibly because a new culture method negatively affected growth and performance. However, subsequent tests without vitamins and minerals confirmed that the trace nutrient addition was not necessary for decolorization to take place.

Tables 4.5 to 4.7 summarize the results obtained from the first seven batch test which defined the basic conditions necessary for fungal decolorization of raw and ozonated ASB effluent to occur. In almost all of the first seven batch tests using raw and ozonated wastewater, *P. chrysosporium* cultures removed a higher percentage of the colour in raw samples compared to the ozonated samples. Furthermore, this higher percentage translated into more significant total colour units removed from the wastewater because the raw samples had higher initial colours. Table 4.7 shows average colour removals per day for attached growth batch tests #3 to #6. Raw colour removals range from 9 to 96 CU/day with an average of 62 CU/day (standard deviation 28 CU/day) while in ozonated samples, the removals varied from to -7 to 33 CU/day with an average of 14 CU/day (standard deviation 15 CU/day) for all three strains of fungus combined. Proportionately, the variability in the ozonated colour removals was much higher than in the raw colour removals.

The standard errors in Table 4.6 and the standard deviations given above show the variability not only among different tests, but between replicates in the same test. Attempts to minimize variability often increased the test inconsistency and made separation of true effects from condition changes difficult.

Table 4.5. Colour changes in raw and ozonated (80 mg/L) ASB effluent during batch tests with and without nutrient addition. Glucose was added at 10 g/L where specified. All samples were buffered to approximately pH 4.5 with potassium hydrogen pthalate and sodium hydroxide. Wastewaters were heat treated before use in decolorization tests.

		Suspended growth test		Attached growth test #1		Attached growth test #2	
		No nutrients	Glucose & nutrients	No nutrients	Glucose & nutrients	No nutrients	Glucose & nutrients
ATCC 24725	Raw	-6%	54%	5%	66%	15%	65%
	Ozonated	-15%	35%	6%	57%	0%	51%
UAMH 3642	Raw	-3%	48%	16%	45%	13%	57%
	Ozonated	2%	30%	27%	49%	35%	43%
UAMH 4521	Raw	2%	61%	8%	8%	11%	62%
	Ozonated	0%	50%	-10%	4%	8%	51%

Initial colour for raw samples ranged from 1100 to 1140 CU and from 460 to 520 for ozonated samples

Table 4.6. Colour changes in raw and ozonated (80 mg/L) ASB effluent during attached growth batch tests #3 to #6. All samples contain 10 g/L glucose and buffer to pH 4.5. Negative values indicate colour increase and positive values show colour reduction. Colour changes are averages of duplicate or triplicate samples with standard error calculated over the duration of the test.

		Attached growth test #3		Attached growth test #4		Attached growth test #5		Attached growth test #6	
		Average	Error	Average	Error	Average	Error	Average	Error
ATCC 24725	Raw	53%	3.4%	6%	0.4%	48%	7.1%	62%	1.3%
	Ozonated	3%	4.5%	-11%	0.7%	39%	0.7%	31%	9.2%
UAMH 3642	Raw	59%	1.1%	28%	0.7%	32%	1.2%	56%	0.9%
	Ozonated	47%	1.7%	1%	0.9%	32%	8.1%	37%	3.8%
UAMH 4521	Raw	46%	7.8%	23%	0.8%	56%	3.0%	18%	0.7%
	Ozonated	48%	0.6%	7%	0.6%	48%	2.6%	-5%	3.7%

Initial colours range from 1100 to 1170 CU on the raw samples and 460 to 480 CU on the ozonated samples.

Table 4.7. Colour removal rate in CU/day in raw and ozonated (80 mg/L) ASB effluent for attached growth batch test #3 to #6. Strains of *P. chrysosporium* used are shown on the left column. All samples contain 10 g/L glucose and buffer to pH 4.5. Negative values indicate colour increase and positive values show colour reduction. Colour changes are averages of duplicate or triplicate samples with the same standard error as shown in the previous table.

	Attached growth test #3, CU/d		Attached growth test #4, CU/d		Attached growth test #5, CU/d		Attached growth test #6, CU/d	
	Raw	Ozonated	Raw	Ozonated	Raw	Ozonated	Raw	Ozonated
ATCC 24725	86	4	9	-7	68	21	91	19
UAMH 3642	96	32	44	1	46	18	82	22
UAMH 4521	75	33	36	-4	80	28	26	-3

4.2.2 Microbial competition

Heat treatment reduced the bacterial, yeast, and fungi populations in the ozonated pulp mill effluent. For the first three batch tests, heat treated wastewater was used to reduce the microorganisms competing for nutrients and oxygen in the decolorization tests. From test #4 on, no heat treatment was used without considerable effect on decolorization. Microbial competition was thus ruled out as a significant factor in impeding decolorization. Populations of microorganisms did increase during the batch tests, as shown in heterotrophic plate counts done at the end of the test and by visual evidence such as clouding of the wastewater. These contaminations were especially prevalent in samples with added glucose and other nutrients.

4.2.3 Comparison of *Phanerochaete chrysosporium* strains

Table 4.8 outlines the performance of the three strains during batch testing of colour reduction on ozonated pulp mill wastewater. The summary data includes attached growth tests #1 through #6 where similar, but not identical, parameters were used, including all tests with nutrients present having 1% glucose added to the sample.

Table 4.8. Comparison of *P. chrysosporium* strains in batch test performance on ozonated (80 mg/L) ASB effluent. Average colour removal included both positive and negative colour changes on the final (7th or 8th day) of the test.

Strain	Maximum removal with nutrients	Maximum removal with no nutrients	Average removal with nutrients	Comments
ATCC 24725	56.9%	5.8%	21.6%	Colour decrease 55% of the time when glucose was present
UAMH 3642	54.3%	34.6%	32.9%	Colour decrease 91% of the time when glucose was present
UAMH 4521	62.8%	9.6%	23.5%	Colour decrease 67% of the time when glucose was present

UAMH 4521 had the highest colour reduction in a single test of all three strains tested. It however did not perform well under conditions without glucose present. The strain was also variable in performance as only 67% of the ozonated samples with glucose present showed the expected colour removals. ATCC 24725 had the next greatest maximum colour removal in the presence of 1% glucose. Similar to UAMH 4521, only a small decrease in colour occurred with no nutrients present. The low proportion of successful colour tests affected the average removal with nutrients, bringing it down to under 22% colour reduction. Although UAMH 3642 achieved the lowest maximum percentage colour removal, the strain was the most consistent in performance with glucose present and was able to reduce effluent colour without the addition of glucose.

4.2.4 Glucose concentrations

Two batch tests, attached growth tests #7 and #8, were run to evaluate the effects of three concentrations of glucose, 2, 4, and 6 g/L on colour degradation by all three strains of *P. chrysosporium* in ozonated pulp mill wastewater. The results are shown in Table 4.9. In general, the ATCC 24725 strain did not achieve good colour removals except at 1% glucose concentration. The UAMH 3642 strain achieved over 30% colour reduction in the 0 and the 0.4% glucose concentrations and greater than 50% removals at the 1% level, but performed poorly at the remaining two concentrations. At the 4, 6, and 10 g/L levels, the UAMH 4521 strain achieved good colour removals of approximately 20 to 50% over eight days. At 0 and 2 g/L, colour reduction was negligible.

Table 4.9. Colour removal with varying concentrations of glucose in ozonated (80 mg/L) pulp mill effluent after 8 days in batch test conditions.

Glucose Concentration g/L		Average colour removal, %		
		ATCC 24725	UAMH 3642	UAMH 4521
0	*	2.5	30.8	-1.0
2	**	2.4	-19.1	7.0
4	**	-2.4	32.1	41.9
6	**	7.1	-14.3	28.9
10	***	36.0	54.3	37.0

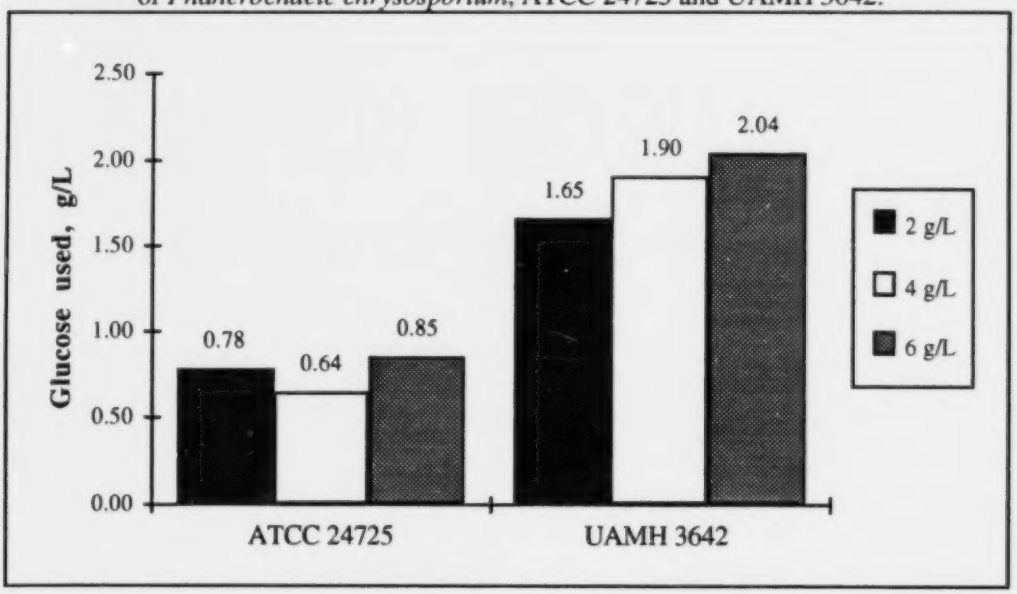
* From attached growth batch test #1 and #2

** From attached growth batch tests #7 and #8

*** From attached growth batch test #5

Similar results were found in glucose concentration monitoring in the batch tests. When two strains, ATCC 24725 and UAMH 3642, were evaluated for average glucose consumption per day, three main features became apparent. Firstly, the quantity of glucose used per day remains approximately the same, regardless of initial concentrations. ATCC 4725 used 0.76 ± 0.11 g/L over 8 days while UAMH 3642 used 1.86 ± 0.20 g/L over 8 days. UAMH 3642 showed a slightly increasing glucose use with higher glucose concentrations, as shown in Figure 4.6, while ATCC 24725 showed no discernible pattern to glucose use. Secondly the two strains utilize the glucose at different rates. For ATCC 24725, the average glucose use ranged from 0.07 to 0.12 g/L/day while in UAMH 3642, the average use was 0.20 to 0.28 g/L. Lastly, glucose use does not appear to be proportional to colour reduction in the samples, because even in samples where colour increased, the glucose depleted was similar to samples where high colour removals occurred.

Figure 4.6. Glucose consumption with varying initial concentrations of glucose in ozonated (80 mg/L) pulp mill effluent after 8 days in batch test conditions with two strains of *Phanerochaete chrysosporium*, ATCC 24725 and UAMH 3642.



TOC was determined for samples from the suspended growth test. These results are included in Appendix D. A high degree of error was present in the analysis because 0.025 M potassium hydrogen phthalate was added to all samples to control the pH and 10 g/L

glucose was added to the nutrient enriched samples. Together these would theoretically add 6400 mg/L TOC; however, the measured value is typically less than the theoretical. Coupled with the original TOC concentration in the wastewater of 240 to 270 mg/L, a TOC of approximately 6650 mg/L could be expected in samples with glucose and 2650 mg/L for the ones without glucose. The higher concentrations also have error associated with dilution of the samples to within the instrument range. Measured values for the initial and final TOC are shown in Table 4.10. No consistent decrease could be seen in the TOC values despite over 50% decolorization occurring in the nutrient enriched samples. No difference among final TOC values for the different strains of fungus were apparent.

Table 4.10. Initial and final TOC values in mg/L for the suspended growth test. Final range values are after 7 to 8 days incubation with different strains of *Phanerochaete chrysosporium*.. KHP is Potassium hydrogen phthalate.

Sample	Condition	Initial TOC mg/L	Final TOC mg/L
Raw ASB effluent treated with fungus	With KHP	2520	2600 to 2670
Raw ASB effluent treated with fungus	With KHP and glucose	6250	6120 to 6330
Ozonated effluent treated with fungus	With KHP	2590	2570 to 2660
Ozonated effluent treated with fungus	With KHP and glucose	6140	6010 to 6230

4.2.5 AOX and colour reduction

AOX was determined in one set of batch studies to compare colour reductions with AOX removals. A strong correlation was found between AOX and colour removal by *P. chrysosporium*, as shown in Figures 4.7 and 4.8. Because colour increased during aeration of the samples, the second graph was based on final colours of the control samples. As more colour compounds are created in the ozonated sample, the fungus has a greater amount to degrade compared to the initial, which was not the case with the raw samples. Making this correction, the AOX and colour removal correlation becomes very similar for both the raw and the ozonated samples, as Figure 4.8 demonstrates, especially within the range of 20 to 60% colour removal. If the correlation is more than happenstance data, then for a measured colour removal, a proportional AOX removal would be expected.

Figure 4.7. AOX and colour removal correlation based on initial colour and final eight day colour for attached growth test #6 Ozonated samples ozonated at 80 mg/L over 30 minutes.

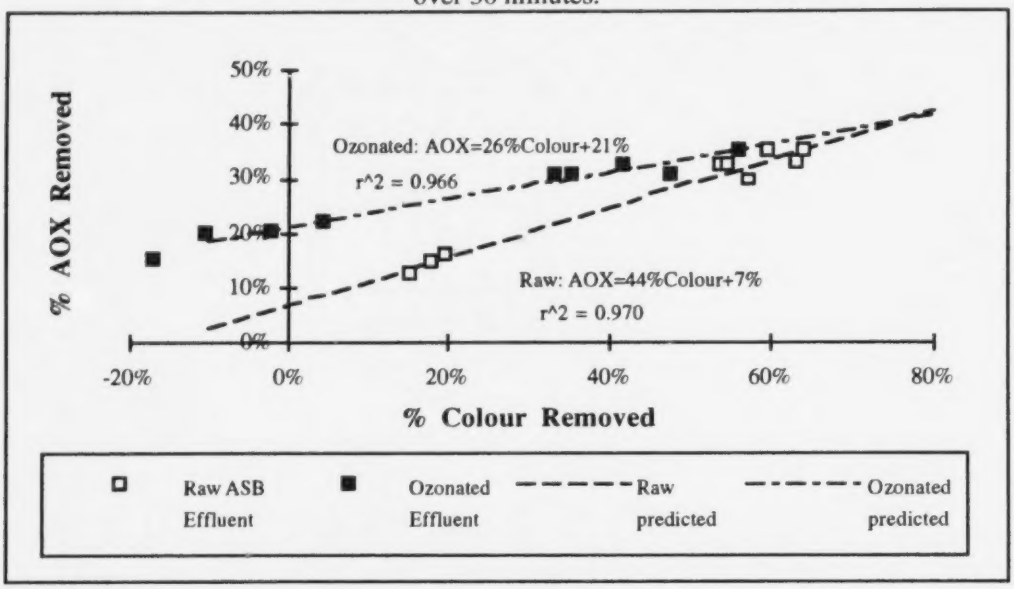
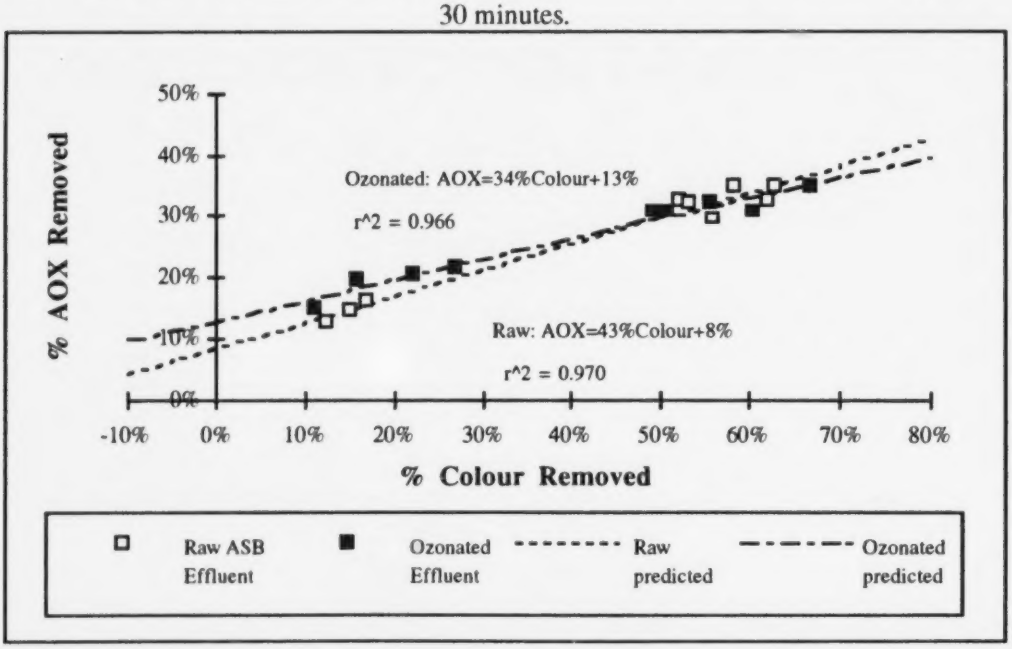


Figure 4.8. AOX and colour removal correlation based on final control colour and final eight day colour for attached growth test #6. Ozonated samples ozonated at 80 mg/L over 30 minutes.



4.2.6 Nitrogen use

Although nitrogen limiting conditions promote decolorization, the possibility that excessive nitrogen starvation occurred during growth and hindered colour removal was investigated in batch tests. Ammonia or ammonium ions were measured both in control samples without fungus and in samples with fungus actively decolorizing the wastewater. These results are shown in Table 4.11. The raw and ozonated control concentrations remained relatively constant within the error range created by the high dilutions necessary to read the concentrations. The ozonated sample consistently showed higher concentrations of ammonia extending from approximately 3.9 to 5.7 mg/L NH_3 - N. Initial concentrations should have been 3.1 mg/L NH_3 - N based on the amount of NH_4Cl added to the wastewater initially. The raw control samples were close to this value, indicating that little or no nitrogen was depleted in the controls during the 8 day incubation. The Limnology laboratory indicated that few substances would interfere with the determination of ammonia except for some heavy metals.

Table 4.11. Nitrogen analysis results for attached growth test #2 for raw and ozonated samples with and without fungus present. All values in µg/L NH_3 - N

	Time, days				
Sample	0	2	4	6	8
Raw Control	4000	4100	3000	2900	NA
Ozonated Control	5000	5700	3900	4800	4900
Raw ATCC 24725	---	34	24	11	36
Ozonated ATCC 24725	---	1.8	[illegible]	20	BDL
Raw UAMH 3642	---	26	[illegible]	18	42
Ozonated UAMH 3642	---	9.4	[illegible]	BDL	BDL
Raw UAMH 4521	---	97	23	24	23
Ozonated UAMH 4521	---	0.3	17	4.9	BDL

NA Not Available because of interferences

BDL Below Detection Limit of 0.18 µg/L

Samples were taken every second day from the biologically treated wastewater. By the second day, all the samples had reached very low levels of ammonia nitrogen, compared to the initial concentrations. Even though the raw samples for each strain of *P.*

chrysosporium tested higher for initial nitrogen concentration, all three strains of fungi did not completely deplete the available ammonia in the raw samples. In the ozonated samples, the nitrogen levels were below the detection limit of 0.18 µg/L by the eighth day in all cases. Reasons for the difference between the depletion of nitrogen in the raw and ozonated samples are unknown. Rapid depletion of the nitrogen in the wastewater may indicate that the fungi were already in a nitrogen deficient environment, as shown by complete use (>99.9%) of the NH_4Cl salt added to the wastewater. The fungi may also require large quantities of nitrogen to begin the decolorization process.

4.2.7 Proteins analysis

Protein analyses were done on the samples from attached growth test #8. The UAMH 4521 strain of *P. chrysosporium* did reduce the ozonated sample colour by up to 51%; however, the protein analysis detected virtually no proteins in the fungi treated samples. The method used, Coomassie Blue protein analysis, may not have been sensitive or specific enough to detect low levels of extracellular enzymes in the fungus treated wastewater.

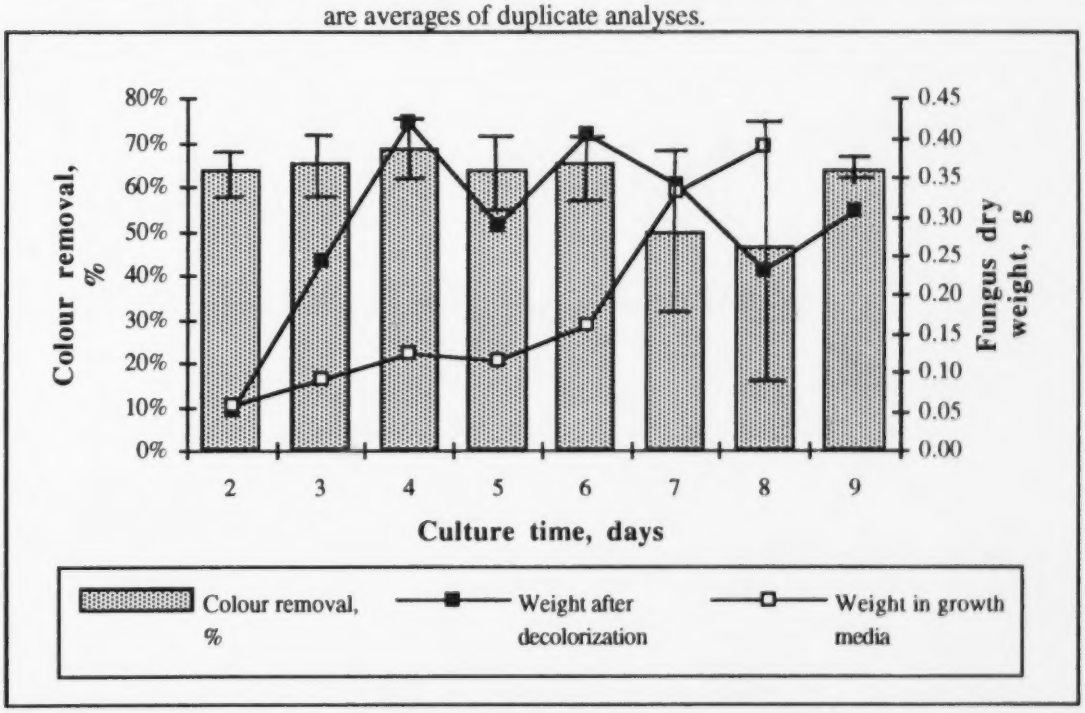
4.2.8 Growth rate test and effect of varying growth periods

Figure 4.9 presents the results from the growth rate test. Using the *P. chrysosporium* strain ATCC 24725, the fungus dry weights were determined every second day for eight days. The growth increased slowly from the initial value to day 6. From day 6 to 8, the greatest amount of growth occurred. The fungus may require longer growth times to increase biomass; however, this does not necessarily correlate with colour removal efficiency, as the next test showed. Another growth rate test run on UAMH 4521 for 21 days showed a similar trend with a relatively large increase in dry weight on day 6 and another around day 15 when the fungus was grown in nutrient medium. Limiting nutrient supplies or waste product accumulation at the longer test times may have slowed growth.

Growth periods were varied from 2 to 9 days in the next test to evaluate what the optimum culture time was before the fungus should be transferred to the wastewater for decolorization. Colour removals and final dry weight of mycelium after eight days decolorization in ozonated (80 mg/L) ASB wastewater are shown in Figure 4.9. High decolorization rates, greater than 50%, were achieved in almost all samples. Decolorization was possible after two days growth in nutrient medium, starting from three week old spore

plates. No improvement in decolorization was found with longer incubation times and the lower biomass produced after decolorization may be a positive aspect of shorter culture times. A correlation may exist between mycelium growth, as shown by dry weight increase, and final colour removals. Day 7 and 8 had slightly lower colour removals and a decrease in mycelial dry weight also occurs. The high growth rate increase shown measure on day 7 and 8 of the growth rate test in nutrient medium correspond to the slight decrease in colour removals. What is not shown in the figure is the variability between duplicate trials. While most of the days showed consistent colour removals between replicates, day 7 and 8 had extremely variable results ranging in colour removals from 31 to 69% on day 7 and 17 to 76% on day 8. These results are shown in entirety in Appendix D. The lower colour removals and final dry weights may be products of this variability without an underlying microbiological explanation. Nevertheless, the variability itself may be caused by a change in metabolism that occurs near that time period and influences both parameters.

Figure 4.9. Effect of culture age on decolorization abilities shown with changes in mycelium dry weight after decolorization and after growth in nutrient solution. All results are averages of duplicate analyses.



Error bars show high and low duplicate values for each colour removal column.

4.2.9 Effect of initial colour concentration

Because raw ASB effluent was typically decolorized by the fungus at a faster rate than the ozonated ASB effluent, colour concentration was investigated as a possible rate controlling factor. Raw ASB effluent was concentrated through freeze drying to 50% of its original volume (twice the initial colour) and then ozonated at approximately 60 mg/L. Concentrated sample properties are shown in Table 4.12. Limited colour removals occurred in the fungus (ATCC 24725) treated samples. Colour reductions ranged from 14 to 30% in the concentrated raw ASB effluent samples and from 11 to 22% in the concentrated ozonated samples. In the raw effluent, the colour of the control sample (without fungus present) decreased by 17% over 10 days while the control for the ozonated samples decreased 3%.

Table 4.12. Characteristics of concentrated raw and ozonated ASB effluent

Parameter	Concentrated ASB effluent	Ozonated concentrated ASB effluent
True colour, CU	2360	1580
TOC, mg/L	470	453
AOX, mg/L	18.2	14.5
BOD, mg/L	27.7	52.4
COD, mg/L	1290	1210

4.2.10 Effect of metals on decolorization by *Phanerochaete chrysosporium*

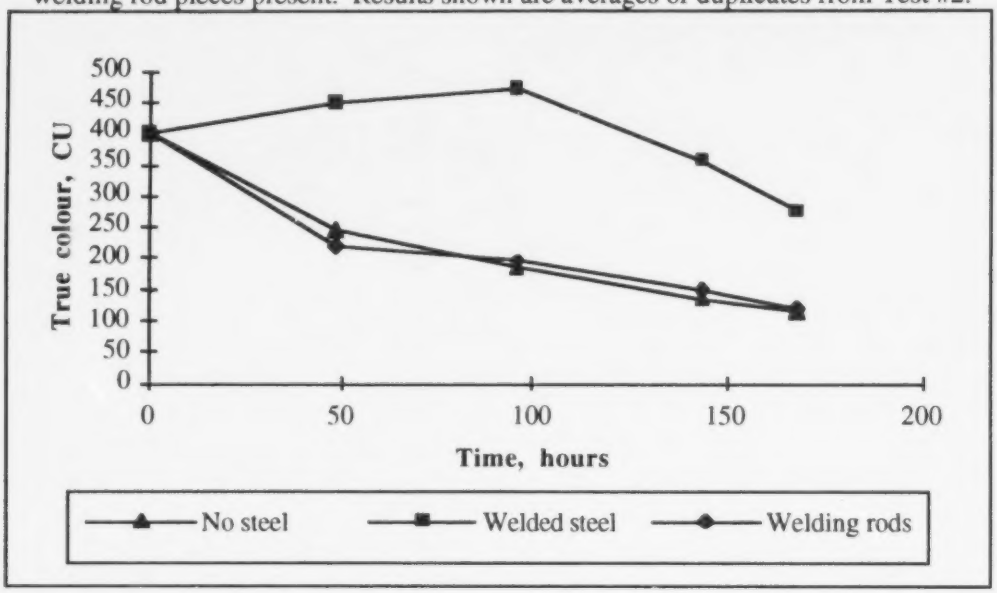
When decolorization occurred during batch tests in glass flasks, but not in the bioreactor, the possibility of metal contamination from the reactors was considered. Steel plates cut from the same grade of steel (304) used in the bioreactor were suspended in the liquid with the fungus. The test conditions and results are shown in Table 4.13. No statistical difference was found among samples in the first test, indicating that the steel plates alone did not affect decolorization. In the second test, samples containing welded steel plates showed substantially lower final colour removals. This result is also shown in Figure 4.10, where the samples containing welded steel plates had a distinct colour change curve, different from all the previous tests. Attempts to repeat these results in Tests #3 and #4 were unsuccessful, as similar colour removals were achieved with or without welded steel plates present. The steel plates were reused from the second test in the third and fourth trials. As corrosion along the weld line was suspected to be a factor in preventing metal

leaching in Test #3, the steel plates were sanded lightly for the last test. Colour removals remained high, 55 to 67%, in the last two tests with no apparent colour removal inhibition. Newly welded steel plates were the only condition that decreased colour removal; subsequent reuse did not display the same results.

Table 4.13. Effect of steel plates on decolorization in batch tests. Tests #1 and #2 average colour removals are results of duplicate trials while Tests #3 and #4 are results of triplicate trials. Initial ozonated effluent colour ranged from 400 to 460 CU.

Test name	Growth conditions	Decolorization conditions	Average colour removal, %
Test #1	Regular conditions	Regular conditions	64.1
Test #1	Steel plates in growth	Steel plates in effluent	63.0
Test #1	Steel plates in growth	Regular conditions	63.0
Test #1	Regular conditions	Steel plates in effluent	62.0
Test #2	Regular conditions	Regular conditions	66.3
Test #2	Welding rod pieces in growth	Welding rod pieces in effluent	62.5
Test #2	Welded steel plates in growth	Welded steel plates in effluent	11.3
Test #3	Regular conditions	Regular conditions	58.3
Test #3	Welded steel plates in growth	Welded steel plates in effluent	54.5
Test #4	Regular conditions	Regular conditions	65.1
Test #4	Welded steel plates in growth	Welded steel plates in effluent	66.7

Figure 4.10. Effect of welded steel plates on decolorization by *Phanerochaete chrysosporium* in ozonated pulp mill effluent over time compared to no metal present and welding rod pieces present. Results shown are averages of duplicates from Test #2.



4.2.11 Metal analysis results

Samples from the fungus treated ozonated wastewater in Test #2 were collected after the end of the six day test. Effluents from the duplicate trials were combined to give one representative sample. Ozonated effluent was also aerated with newly welded steel plates at pH 4.5 and at 37°C to leach out metals from the welded steel plates without biological interferences from the fungus. These two samples, plus the initial ozonated sample, were analyzed for the following nine metals: aluminum, cadmium, cobalt, chromium, copper, iron, nickel, lead, and zinc. Cobalt, lead, and zinc concentrations were negligible in all three samples tested. Aluminum concentrations ranged from 1.63 to 2.15 mg/L, but these readings may have been elevated by calcium in the sample which has an emission similar to aluminum. The aluminum values were similar among the three samples and were not considered to be responsible for any inhibition effects. Concentrations of the remaining metals are shown in Table 4.14.

Table 4.14. Metal concentrations in initial ozonated, welded steel and fungus treated, and welded steel and aerated ozonated ASB effluent samples. Values were measured by an Inductively Coupled Plasma (ICP) instrument and are given as mg/L of the metal.

Metal mg/L	Initial ozonated sample	Fungus treated ASB effluent in presence of welded steel plates	Welded steel plates aerated in ozonated ASB effluent
Chromium	0.000	0.378	0.000
Copper	0.000	0.047	0.001
Iron	0.209	7.99	0.861
Nickel	0.000	1.53	0.237

To test which metal, if any, had negative effects on *Phanerochaete chrysosporium* decolorization of pulp mill wastewater, ozonated ASB effluent was spiked with metal sulphates and incubated with the fungus. Each metal was tested individually at 30% higher concentrations than found in the metal analysis and all metals were tested together to evaluate whether interactive effects occurred. Samples were taken every second day for six days. Glucose (10 g/L) was added but the wastewater was not buffered. If the pH fell below 4.0, NaOH was added to bring the pH back up. Two batch tests with spiked metals were run. The first had metal added only to the wastewater during the decolorization stage, while the second had metals added to both the growth and decolorization stages. Table 4.15 shows the metal compound and the concentration of metals added to either or both growth solution or ozonated ASB effluent.

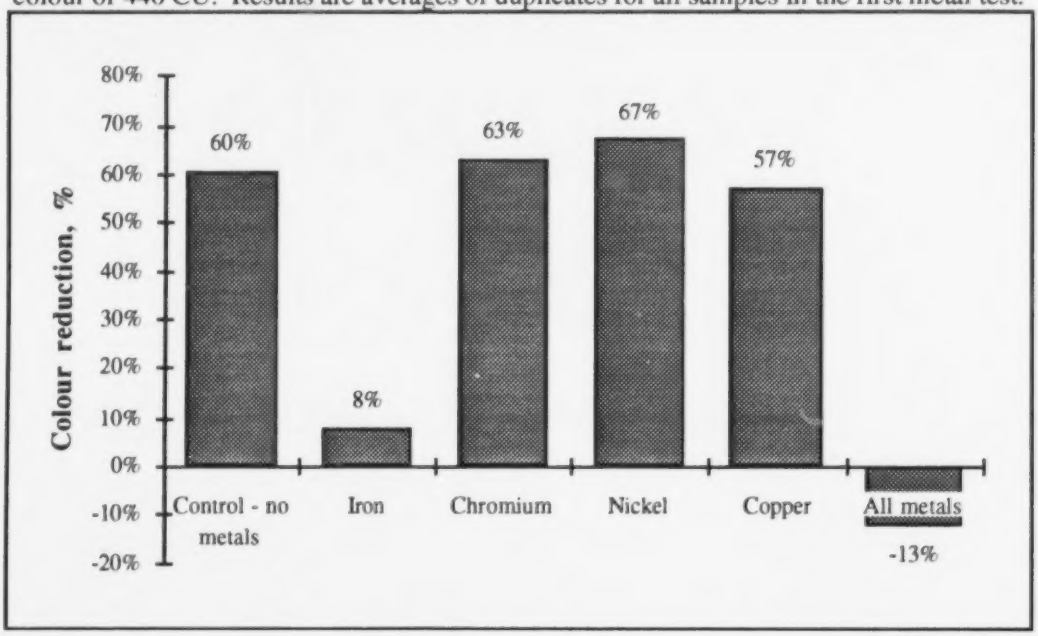
Table 4.15. Metal concentrations and compounds added to growth solution and/or ozonated wastewater to test inhibition effects of specific metals individually and all together.

Metal	Form added	Probable ion present	Concentration, mg/L
Chromium	$Cr_2(SO_4)_3 \cdot 6H_2O$	Cr^{3+}	0.5
Copper	$CuSO_4 \cdot 5H_2O$	Cu^{2+}, Cu^{3+}	0.06
Iron	$FeSO_4 \cdot 7H_2O$	Fe^{2+}, Fe^{3+}	10.5
Nickel	$NiSO_4 \cdot 6H_2O$	Ni^{2+}	2.0
All metals	all the above	all the above	all the above

Results from the first metal test (Figure 4.11) indicate that iron may inhibit decolorization and that all metals added together have a greater effect than iron alone. These results are

shown in Figure 4.11, where the control without metals, the chromium, copper, and nickel spiked samples all reduced colour by 57 to 57% after six days. In contrast, the sample with added iron only reduced colour by 8% and the sample containing all metals increased the colour by 13%. Some colour increase in the iron and all metals spiked samples was attributed to metal complexes, such as iron chloride. In the second test, this was accounted for by running controls with metals but no fungus present to measure colour increase with aeration of pulp mill effluent. Complete data tables for the metal tests are given in Appendix F.

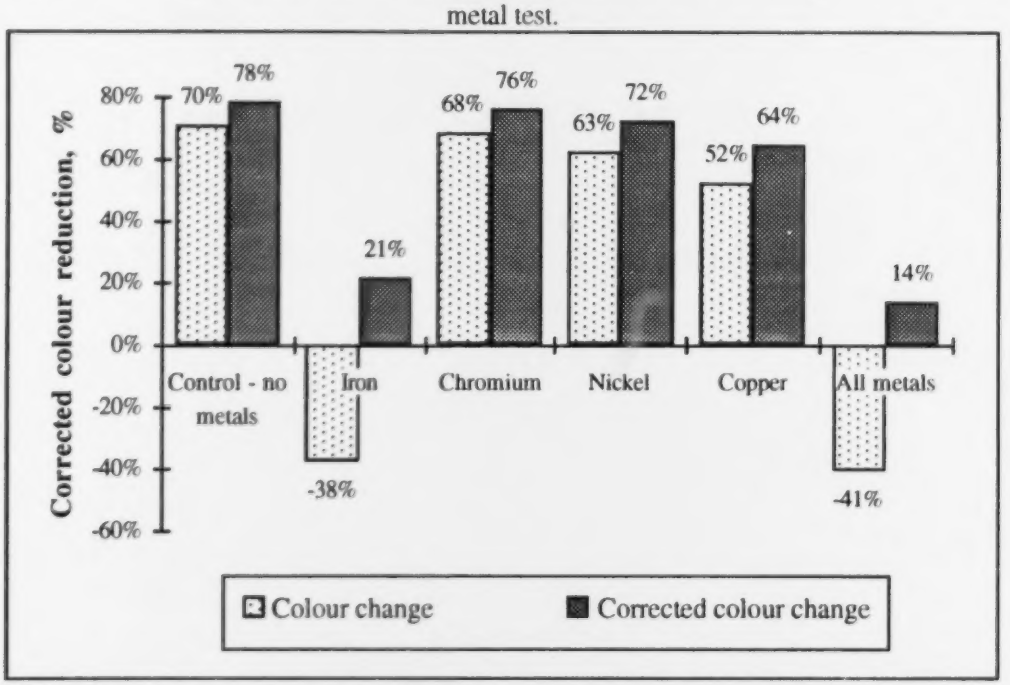
Figure 4.11. Colour change after six days with metal spiked ozonated ASB effluent incubated with *P. chrysosporium*. Negative values show colour increase from the original colour of 440 CU. Results are averages of duplicates for all samples in the first metal test.



Metals were added to both the growth solution and the ozonated ASB effluent in the second metal test. Also controls were run to determine colour increase of the ozonated wastewater with no metals, iron present, and all metals added. Figure 4.12 shows the results of this test. Corrected removals use the final values from the colour increase controls as the initial colour to determine overall percentage colour change. With the correction, the colour removal increases in all samples because the final colour of colour increase controls with

and without metals are higher than the initial ozonated effluent colour. A similar pattern to the previous colour test is apparent. With the no metals, chromium, copper, and nickel samples, colour removal ranged from 64 to 78% (52 to 70% excluding colour correction) while the fungus only removed 21% and 14% in iron spiked and all metals samples. Without accounting for the colour increase associated with the metals themselves, both samples would have had negative colour removals, or colour increases, in other words. Copper may have a small negative effect on colour, because in both tests, the final colour removal for copper spiked samples was not as high as for the control or the samples with nickel and chromium present. ANOVA analysis run on both tests showed that the variability between the different metal treatments was much greater than variance between replicated trials.

Figure 4.12. Colour change after six days with metal spike ozonated ASB effluent incubated with *P. chrysosporium*. Negative values indicate a colour increase from the original colour of 440 CU. Results are averages of duplicates for all samples in the second metal test.



Corrected colour change is calculated using the final colours of the corresponding colour increase controls. Colour change with no correction is based on the initial colour of the ozonated sample.

4.2.12 Improved medium evaluation

Improved medium was evaluated in both raw and ozonated samples to determine if colour reduction without glucose present was enhanced. Complete data for this set of tests is given in Appendix G.

For comparison with previous work, 10 ± 0.5 g wet weight of fungus was added to raw and ozonated ASB effluent containing 10 g/L glucose. After 24 hours, 16% and 7% colour removals were achieved in the raw and ozonated effluents, respectively, with improved medium added to the wastewater. After 7 days, 73% of the original colour in each of the raw and ozonated samples had been removed. The final wet weights showed that the fungus had almost doubled in mass after 7 days.

Next, colour change in improved medium was compared to glucose supplemented medium. Duplicate trials were set up for each condition shown in Table 4.16. Final colour removals show that although fungus with improved medium reduced colour substantially after 7 days, colour reduction in ozonated samples was still higher with suspended growth fungus and glucose as a carbon source. Improved medium tests also had the most variability in results between duplicate trials. Attached growth fungus again showed better removals, even with 30 to 40 % less mass of fungus present.

Table 4.16. Improved medium comparison with glucose supplemented wastewater in raw and ozonated ASB effluent. All results are averages of duplicate colour change after 7 days using approximately 10 g wet weight of fungus in 150 mL wastewater. Initial raw colour was 1220 CU and initial ozonated colour was 460 CU.

	Raw ASB effluent	Ozonated ASB effluent
Improved medium, no glucose	47% ± 6.7%	33% ± 11.8%
Suspended growth with 10 g/L glucose	34% ± 0.3%	49% ± 2.2%
Attached growth with 10 g/L glucose	67% ± 3.2%	55% ± 0.8%

When this test was repeated using UAMH 3642 instead of ATCC 24725, similar results were obtained where colour in the raw wastewater with improved medium was reduced by 55%, compared to 52% reduction in the ozonated with glucose samples.

Tables 4.17 and 4.18 present the factorial analysis results for the tests using ozonated and raw wastewater, respectively. The ozonated factorial test had high variability in results with standard error of 6.5% for the mean and 13% for effects. This made any interpretation of results difficult because the high error may have masked significant effects or interactions. Of the main effects, buffer negatively affected colour removals, decreasing the colour reduction at higher levels of buffer. Manganese had the opposite effect, but not as strongly as compared to the buffer. Higher levels of manganese promoted decolorization. Interactions were negligible compared to the standard error.

Use of raw wastewater and attached growth fungus yielded less variability in results. Table 4.18 shows the main effects and interactions, in both percentage removals from the initial colour and final colour units calculated from the 2^3 factorial test. Increasing the Tween 80 to the highest level increased the colour reduction by almost 4% or 45 CU. Manganese also had a positive effect on colour reduction, causing 5% or 58 more colour units to be removed at the high level. Unexpectedly, the buffer had a negative effect on the fungus by reducing the overall colour removals by 23.5% or 270 CU when the concentration was increased from none to the high level. Part of this inhibition may be negated with manganese addition, as shown by the interaction between these two parameters. Increasing both buffer and manganese to the high level caused a 6.1% colour reduction (18 CU) from the base level. Other interaction effects appear to be negligible.

Table 4.17. Evaluation of improved medium using ATCC 24725 in ozonated wastewater. Final main effects and interactions are based on 7 day colour change. Ozonated ASB effluent initial colour was 400 CU. Percentages given are colour removals based on the initial colour, while colour values given are colour change from the original.

Effect	Effect in % colour reduction	Effect colour unit change CU
Mean	14.5% ± 6.5%	-58 ± 26
Tween 80	-5.3% ± 13%	21 ± 52
Buffer	-56.6% ± 13%	226 ± 52
$MnSO_4$	24.7% ± 13%	-99 ± 52
Tween 80 x Buffer	-15.9% ± 13%	64 ± 52
Tween 80 x $MnSO_4$	-7.2% ± 13%	29 ± 52
Buffer x $MnSO_4$	-0.9% ± 13%	4 ± 52
Tween 80 x Buffer x $MnSO_4$	-17.8% ± 13%	71 ± 52

	Low	Medium	High
Tween 80	0.10 mL/L	0.50 mL/L	0.90 mL/L
Buffer, KHP + NaOH	None	0.025 M KHP	0.050 M KHP
		0.004 M NaOH	0.008 M NaOH
$MnSO_4 \cdot H_2O$	0.1 mM	0.5 mM	0.9 mM

Table 4.18. Evaluation of improved medium using ATCC 24725 in raw wastewater. Final main effects and interactions are based on 7 day colour change. Raw ASB effluent initial colour was 1150 CU. Percentages given are colour removals based on the initial colour, while colour values given are colour change from the original.

Effect	Effect in % colour reduction	Effect colour unit change CU
Mean	66.6% ± 0.4%	-766± 4.9
Tween 80	3.9% ± 0.9%	-45 ± 9.8
Buffer	-23.5% ± 0.9%	270 ± 9.8
$MnSO_4$	5.0% ± 0.9%	-58 ± 9.8
Tween 80 x Buffer	-0.7% ± 0.9%	8 ± 9.8
Tween 80 x $MnSO_4$	-0.4% ± 0.9%	5 ± 9.8
Buffer x $MnSO_4$	6.1% ± 0.9%	-70 ± 9.8
Tween 80 x Buffer x $MnSO_4$	1.5% ± 0.9%	-18 ± 9.8

4.3 Colour change after ozonation

During the batch study experiments, controls were set up under the same conditions as the test samples, except they did not contain any fungal cultures. At 37 °C, with 100% oxygen bubbling through the wastewater, these controls showed from 0 to 31% colour increase after eight days on the ozonated samples. The raw, unozonated wastewater used as controls did not show significant colour increases; in most cases the colour decreased from the original. A combination of relatively high temperatures and saturated oxygen conditions may have been responsible for the colour increase. To investigate this assumption, a test was carried out in triplicate at three temperatures, 4 °C, 22 °C, and 40 °C, with oxygen bubbled through 1.8 L of ozonated (80 mg/L) pulp mill wastewater.

The wastewater used was from the ASB in May after operation had resumed after shutdown. This effluent did not have as high initial colour as the wastewater used in the initial batch studies. When ozonated with 80 mg/L ozone at room temperature, the resulting colour was 100 to 200 CU lower than was typically achieved with the previous samples. As the lower colour may have affected the results of this test, it was repeated later with wastewater samples from the ASB taken in April, 1995 before shutdown occurred.

No buffer, glucose, or nutrients were added to the wastewater, nor was the pH adjusted to 4.5. Instead the pH was left at the initial value, approximately 7.5, and allowed to increase or decrease over time in the samples. Table 4.19 shows the average colour results from three samples at each temperature on the initial and final (10th) day of the test. Appendix E contains the data from the colour change tests.

Table 4.19. Colour results from the ozonated (80 mg/L) colour change test, trial #1. All values of true colour are in CU. Values represent an average of triplicate samples.

Test Series Day	Time, hrs	Colour at 4 °C	Colour at 22 °C	Colour at 40 °C
0	0	320	320	320
10	240	287	330	353

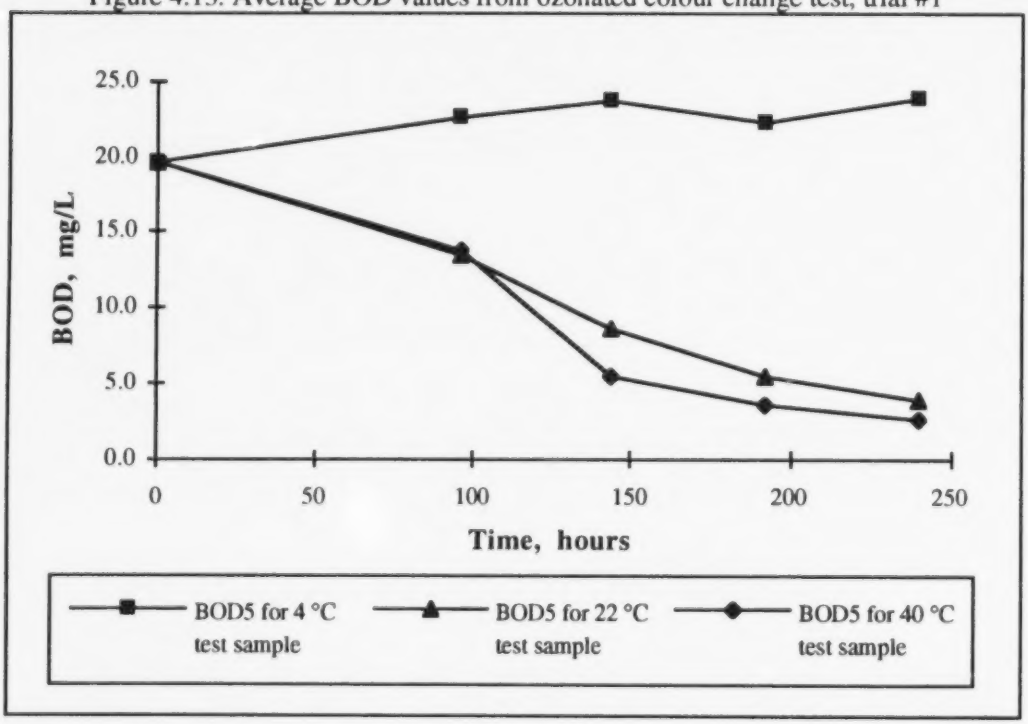
At 4°C, a colour decrease occurred. Substantial dark brown coloured precipitate was present at the bottom of the 4°C flasks. Colour decrease after 10 days amounted to between 9 to 13%. In the 22 °C replicates, the colour increased approximately 3% (10 CU) over the 10 days of the test. This amount is insignificant and is well within the experimental error

of the colour analysis. At 40 °C, the approximate temperature at which the batch tests were run, a greater colour increase occurred. The final colours were 30 to 40 CU (9 to 13%) higher than measured in the initial samples.

The peak colour was generally reached within three days, then declined slightly to the final value. Evaporation was not largely responsible for colour increases as the evaporation controls showed negligible evaporation rates of about 0.6 to 1.1% at the temperatures evaluated.

BOD was also measured on every second day of the test. The average results from the triplicate samples are shown in Figure 4.13.

Figure 4.13. Average BOD values from ozonated colour change test, trial #1

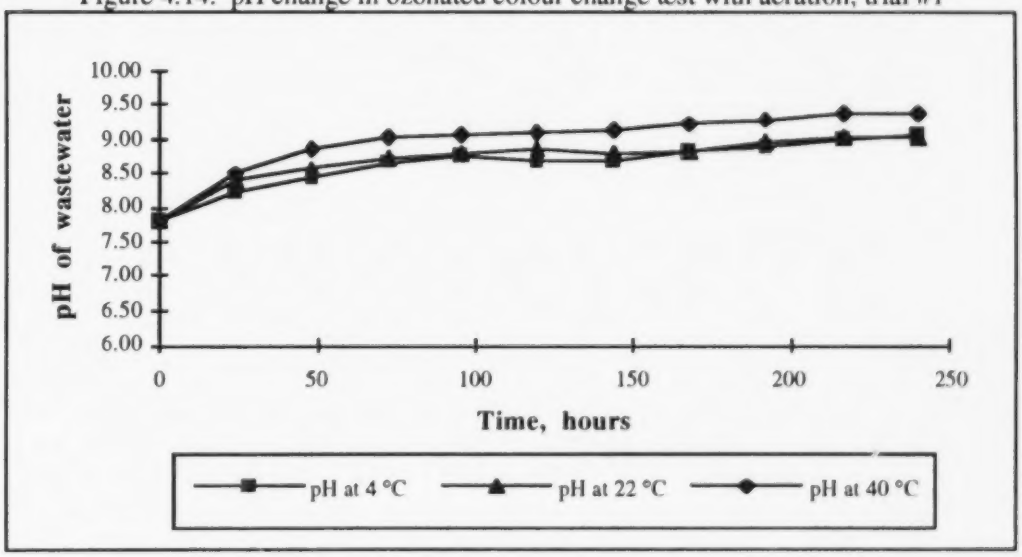


As both the data and the graph show, the BOD decreases quickly at the two higher temperatures, 22° and 40°C, while increasing slightly in the 4°C samples. The biological activity, as indicated by the HPC's, increased in both of the higher temperature samples but remained constant at 4°C. It was not possible to separate what role, if any, the oxygenation

and colour increase had on BOD change. The two options for limiting microbial growth were filtering through a 0.45 µm filter or heat sterilizing; these options were undesirable because they would have changed the characteristics of the wastewater and interfered with the test.

The sample pH was not adjusted initially to the same pH as the batch tests were run at, approximately 4.5 pH units. In monitoring the pH of the oxygenated samples daily, the pH was found to increase in all samples, as shown in the Figure 4.14. During the initial batch tests, some increase in pH was found to occur immediately after ozonation, without any aeration, possibly caused by reactions continuing for a few hours in the ozonated pulp mill effluent. The increase was similar at 4° and 22°C and about 0.3 pH units higher at 40°C. Although the mechanism that causes the pH increase is not known, oxygenation may induce hydroxyl functional groups to be shed off the main compounds.

Figure 4.14. pH change in ozonated colour change test with aeration, trial #1



A second trial was set up using a factorial design to determine what effect temperature and initial pH have on colour increase of an ozonated sample with oxygen bubbled through it. Duplicates at two levels, plus centre point triplicates were run in order to separate the main effects and interactions. Results of the colour tests in the factorial experiment for the second trial of the ozonated colour change test are shown in Table 4.20 and the statistical analysis in Table 4.21.

Table 4.20. Factorial design and colour results for trial #2 with aerated ozonated (80 mg/L) wastewater. All results are final (10 day) true colours in CU.

Low temperature		Mid temp	High temperature	
Low pH	High pH	Mid pH	Low pH	High pH
390	430	470	620	470
410	430	470	590	460
		470		

Initial colour = 460 CU

Low initial pH = 4.0 Low temperature = 4 °C

Mid initial pH = 5.6 Mid temperature = 22 °C

High initial pH = 7.2 High temperature = 40 °C

Table 4.21. Analysis of variance for temperature, pH, and interaction effects from second colour change test

Source of Variation	Effect, CU	Sum of Squares	Degrees of freedom	Mean Square	F	Significant at 1%?
Temperature	120	28800	1	28800	164.6	yes
pH	-55	6050	1	6050	34.6	yes
Temperature x pH	-85	14450	1	14450	82.6	yes
Error within treatments		700	4	175		
Totals		50000	7			

Both main effects and the interaction between temperature and pH were found to be highly significant at the 1% level. Relative to the standard error of 9.4 calculated from the centre points, all three effects calculated influenced the colour more than can be attributed to experimental error alone. Temperature had the strongest effect on the colour of the samples where an increase in temperature increased the mean final colour of 475 CU by about 120 CU. pH had a smaller average effect with a higher pH causing less of a colour change. However, the effects of temperature and pH should not be interpreted separately because of

the large interaction between the two. The interaction arises from a difference in sensitivity to pH change for the higher and lower temperatures.

BOD_5 was again monitored in the second trial on days 0, 2, 6, and 10. The results of the final 10 day BOD_5 are presented in Table 4.22 with the ANOVA following in Table 4.23.

Table 4.22. Factorial design and BOD results for trial #2 with aerated ozonated (80 mg/L) wastewater. All duplicate and triplicate results are final (10 day) BOD_5 in mg/L

Low temperature		Mid temp	High temperature	
Low pH	High pH	Mid pH	Low pH	High pH
27.04	28.91	2.87	18.15	4.46
27.52	30.06	2.95	16.31	4.30
		2.65		

Initial colour = 460 CU

Low initial pH = 4.0 Low temperature = 4 °C

Mid initial pH = 5.6 Mid temperature = 22 °C

High initial pH = 7.2 High temperature = 40 °C

Figure 4.15 BOD_5 results for trial #2 with aerated ozonated (80 mg/L) wastewater. Factorial test setup uses three temperatures and pH levels. Results are total BOD_5 in mg/L.

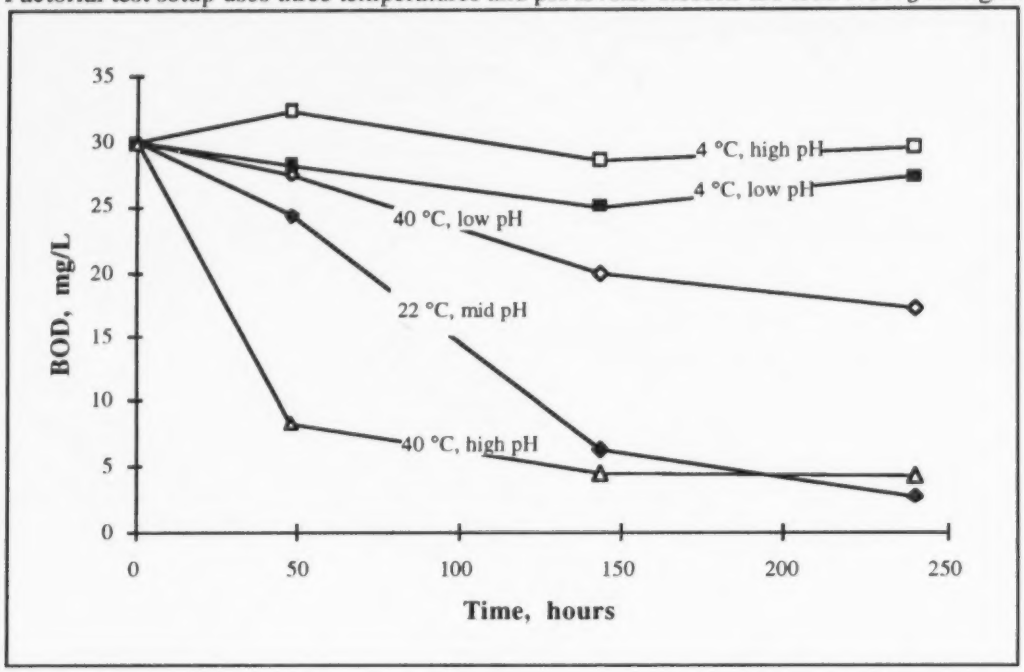


Table 4.23. ANOVA for trial #2 BOD results with aerated ozonated (80 mg/L) wastewater

Source of Variation	Effect, BOD mg/L	Sum of Squares	Degrees of freedom	Mean Square	F	Significant at 1%?
Temperature	-17.58	617.94	1	617.94	995.85	yes
pH	-5.32	56.66	1	56.66	91.31	yes
Temperature x pH	-7.53	113.33	1	113.33	182.63	yes
Error within treatments		2.48	4	0.62		
Totals		790.40	7			

All three sources of variation were significant compared to error within the treatments. Temperature again showed the largest effect on final BOD of the samples, but the effects of pH and more importantly, the interaction between pH and temperature must also be considered. Higher temperatures and higher pH favour the reduction of BOD in the aerated ozonated pulp mill effluent. Little reduction in BOD occurred at 4°C at either pH tested. At

40°C and a low pH, the BOD was only reduced by about 13 mg/L over 10 days, possibly because microbial activity is reduced under acid conditions. At mid and high pH and temperatures, the greatest reduction in BOD occurred, 26 to 28 mg/L at the end of the ten day test. Both these conditions favour the growth of bacteria naturally present in ASB effluent and would be expected to decrease the available BOD rapidly under the completely oxygenated environment.

Soluble BOD was determined on the final day of the test to evaluate whether the precipitated material affected the BOD analysis, i.e. whether the soluble BOD differed from the total BOD measured on the previous days. At higher pH's, greater quantities of precipitates or suspended solids must have been present that contributed to total BOD, but were removed in the soluble BOD test. At 40°C and a high pH, where the lowest BOD occurred, the greatest microbial population could be expected. Filtering through a 0.45 μm filter would have removed much of the bacteria that would have contributed to an increase in BOD, as measured in the total BOD sample. At low temperatures, the presence of insoluble material in the sample had little effect on BOD even though the highest quantity of visible solids was present in these samples.

During the ozonated colour change tests, the pH was also monitored. Samples with the initial pH adjusted to 4.0 did not change significantly. The mid pH, 5.6 based on the initial pH reading after ozonation, increased in pH the most, up to 8.3 units. The high pH increased from 7.2 to 9.3 in 10 days.

4.4 ASB pulp mill effluent characteristics

Samples for this study were taken from the second cell of the ASB at the Weyerhaeuser Canada Ltd pulp mill in Grande Prairie, Alberta. Sample characteristics, in terms of water quality parameters, were measured each time fresh sample arrived and after storage to determine possible changes. Table 4.24 shows the general parameter range on raw and ozonated ASB effluent used in testing. Plant shut down had significant effects on sample characteristics because large volumes of water were used to clean equipment and sewers without the normal contaminant load from pulping. Low ranges in Table 4.24 usually represent a sample shipment that was received after production resumed following shut down.

Table 4.24 Characteristics of raw and ozonated (80 mg/L over 30 minutes) ASB pulp mill effluent used in testing.

Parameter	Units	Raw	Ozonated
TOC	mg/L	223 to 264	211 to 241
TIC	mg/L	95 to 101	88 to 90
TC	mg/L	323 to 361	301 to 331
AOX	mg/L	7.96 to 9.64	5.55 to 6.82
COD	mg/L	560 to 676	496 to 562
BOD	mg/L	12.0 to 20.4	19.6 to 51.3
True colour	CU	950 to 1250	400 to 520
48 hr HPC @35 °C	cfu/mL	$3x10^5$ to $19x10^5$	$1.7x10^4$ to $1.7x10^5$
7 day HPC @20 °C	cfu/mL	$11x10^5$ to $59x10^5$	$33x10^4$ to $25x10^5$

5. DISCUSSION

5.1 Immobilized fungi - rotating biological contactor

When both the Plexiglas and the stainless steel bioreactors failed to remove colour from pulp mill wastewater, many potential causes were explored. Microbial contamination was investigated, both through characterizing the reactor microbial populations, and through reduction of potential competing microorganisms by heat treatment. While some testing used heat or filtration to sterilize the effluents before use (Eaton *et al.* 1982; Kirk *et al.* 1986), the majority of decolorization studies do not attempt to control microbial populations in pulp mill effluent. However, a significant difference exists between alkaline extraction (E_1) stage effluent used in most studies and the secondary treated combined effluent in this work. E_1 effluent has come from a high temperature, high pH stage of wood pulping where microbial populations are fairly low due to the adverse conditions. ASB effluent used in this study was combined effluent that had already been biologically treated for 10 to 14 days. Neutral pH, added nutrients, and high aeration are all needed to biodegrade the wastewater, but the increase in heterotrophic bacteria, yeasts, and fungi was thought to interfere with decolorization in the bioreactors.

Ozonated ASB effluent has a greater BOD than the raw wastewater, but the amount of easily biodegradable organics is still limited. The original theory relied on the ozonation step to produce enough available carbon for fungal degradation without a supplemental carbon source. If other microbes consume this substrate before the *P. chrysosporium* can use it for degrading colour causing compounds, then an extra carbon source may become necessary.

The MyCoR reactor developed at North Carolina State University does not require effluent sterilization before use (Campbell *et al.* 1982; Pellinen *et al.* 1988; Yin *et al.* 1989a; Yin *et al.* 1989b) and does not seem to suffer from microbial competition. *P. chrysosporium* has been reported to be a fast growing fungus that dominates high temperature, low pH bioreactor systems without interference from other microorganisms (Campbell *et al.* 1982). Some bacteria and yeasts have been shown to have synergistic effects with some white-rot fungi, increasing fungal growth by 200% and lignin decay by 200 to 1000% over the basidiomycete grown alone (Blanchette and Shaw 1978). As *P. chrysosporium* was not included in this study, no direct conclusion can be made about its association with other microorganisms. Axenic cultures of fungus do not necessarily improve degradation. Bacteria possess some abilities to biodegrade complex organics but often toxicity becomes a controlling factor (Barr and Aust 1994). With white-rot fungi, toxicity of pulp mill

effluent is reduced and large compounds are fragmented into smaller, more bioavailable forms that bacteria can attack further. Most evidence suggests that a combined system with both bacteria and fungi present would be most successful in degrading pollutants.

Microbial contamination seems an unlikely culprit for the poor bioreactor colour results. In batch tests, without heat treatment or other sterilization procedures of the ASB effluent, colour reduction occurred even when a mixed population was known to be present from HPCs and visual observation of effluent clouding indicating bacterial growth.

If reducing bacterial populations had been necessary, the combined ozonation/fungal process would have greatly increased in cost for a larger scale system. Ozonation has to take place after an initial biological treatment to increase the available carbon; yet to virtually sterilize the pulp mill effluent after the ASB would require prohibitive quantities of energy and would complicate the process even more by requiring a cooling system to bring the temperature back down to the optimum for *P. chrysosporium*.

Lack of available carbon was ruled out because even with 10 g/L glucose in the bioreactors, no colour reduction occurred. In similar batch test situations, colour reduction almost always took place within 24 hours. Even when the bioreactor was run at a 72 hour HRT or as a batch system, no colour reduction was found. High evaporation rates in the Plexiglas reactor could have concentrated both colour and solids in the reactor and affected performance but this was not a significant factor in the stainless steel bioreactors when evaporation was kept below 5% of the reactor volume over 24 hours.

A sufficient oxygen supply is necessary for lignin degradation and colour removal by *P. chrysosporium* (Kirk et al 1978; Yang *et al.* 1980; Eaton *et al.* 1980). Samples from the stainless steel bioreactor had measured oxygen concentrations greater than 7 mg/L showing that oxygen was not a limiting factor for colour removal.

Agitation, considered by some researchers to adversely affect lignin degradation and colour removal (Kirk *et al.* 1978; Yang *et al.* 1980; Kirk 1985) was limited in the bioreactors because the rotation speed was very slow at 1 to 2 rpm. With the MyCoR reactor, reduced decolorization rates were reported at disc speeds of 7 to 18 rpm, but even at these velocities, colour reduction was not completely negated. With the IF-RBC systems, stopping the drive motor and allowing the sponges to remain stationary did not improve

decolorization although a thick layer of fungal biomass was attached to the bottom of the immersed sponges, showing good fungal growth.

When the Plexiglas bioreactor was operated previously these difficulties in obtaining colour reduction were not observed (Mao 1996). With 80 mg/L ozone and 24 hours retention time, the ozone/fungal process simultaneously achieved final effluent quality of approximately 200 CU colour and 3.15 mg/L TOX. These results are shown in Figure 5.1. Because of process modifications at the Weyerhaeuser mill where ASB effluent samples were obtained, initial colour concentrations were usually higher (≈10 to 15%) in the previous study. AOX concentrations were approximately the same. This small decrease in initial colour should not have had substantial effects on the ozone/fungal process.

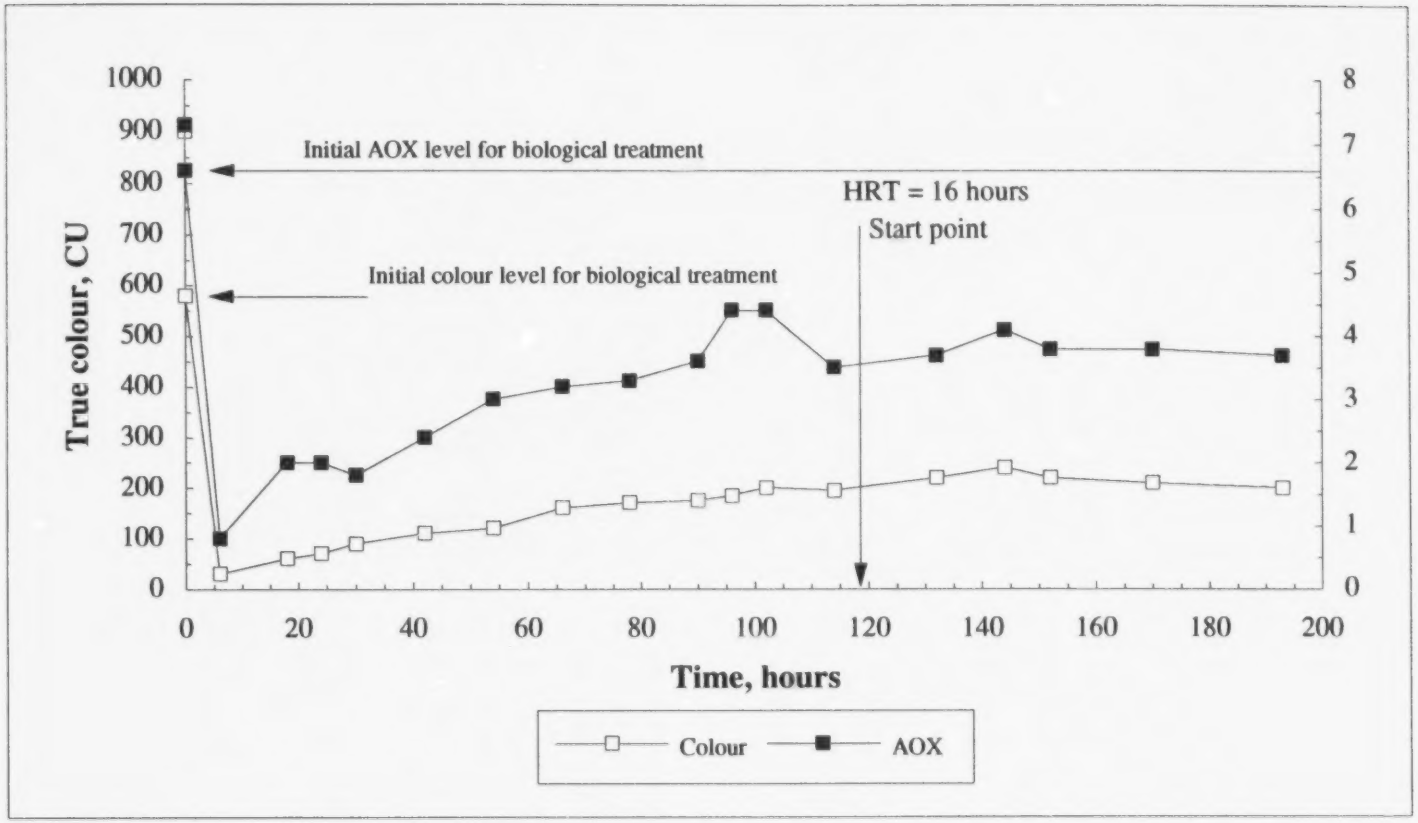


Figure 5.1. Colour and TOX removal using ozone/fungal continuous flow bioreactor system. HRT was 16 and 24 hours and ozone dose was 80 mg/L. Adapted from Mao (1996).

Finally, the reactor material was suspected as it was one of the few differences between the batch and bioreactor systems. In June 1995, nutrient solution that had been recycled through *P. chrysosporium* inoculated stainless steel reactors was analyzed for glucose and for trace metals. High levels of nickel and iron, 8.82 mg/L and 0.885 mg/L respectively, were found in the used nutrient solution, while only about 10% of the original glucose (10 g/L) had been consumed. Corroding air lines were thought to be responsible for metal leaching and for the subsequent effect on fungus growth and decolorization. Even after the air lines were replaced with new stainless steel tubing lines instead of the softer tubing that originally was used, no improvements took place.

Steel testing in the batch systems was seen as a way to test bioreactor conditions selectively with procedures that were known to yield colour reductions. When 11% colour removals occurred only with welded steel coupons and greater than 60% colour reductions with just the steel coupons or welding rod pieces, welded steel was considered responsible for poor bioreactor performance. However, this result did not indicate what factor in the welded steel was the actual problem. Samples of the treated effluent with welded steel coupons present were analyzed for trace metals as shown in Table 4.13. The higher levels of metals in the fungus treated effluent compared to the effluent that had been aerated with welded steel coupons and no fungus present were unexpected. Metals were anticipated to be adsorbed or bound at least in part to the fungal biomass, because of the known metal adsorptive capacities of fungi and other microorganisms (Kapoor and Viraraghavan 1996). Considering a digestion or extraction was not performed on the fungal biomass to remove metals, lower concentrations were expected in this sample. Instead, the fungus treated samples had up to 47 times higher, in the case of copper, concentrations of the metals than found in the sample without fungus. Both of these samples had higher metals concentrations than the initial ozonated sample. Trace metals, including iron and copper, had been added to the nutrient solution for fungus pregrowth. However, even if the fungus had stored all iron and nickel in the nutrient solution it was grown in, then released it into the effluent during decolorization, this would only account for less than 1% of the metals found during the ICP analysis.

These results might be explained by pH changes the fungus creates. *P. chrysosporium* has been found to produce organic acids (Barr and Aust 1994) during growth and decolorization. These acids may have increased the metal leaching or corrosion compared to the sample without fungus where the pH increased slightly from 4.5 to greater than 5 pH units during aeration. If the fungus did adsorb metals, then the actual concentrations

leached from the welded steel plates may have been greater than actually measured. This factor was accounted for by increasing the spiked concentration by 30% when the metals were tested individually and in combination.

In two tests with added chromium, copper, iron, and nickel, the same results were found. Iron reduced the amount of decolorization to 21%, even when colour increase from the added iron itself was taken into account. When all metals were added, decolorization was less than in the iron samples, but not by a large amount as 14% of the colour was removed. Chromium, copper and nickel spiked samples had no statistical difference from the control sample with no metals present, ranging in corrected colour reduction from 64% (copper) to 78% (control). Iron may interfere with a metabolic pathway that *P. chrysosporium* uses to decolorize pulp mill effluent. Growth of the fungus, from visual observations, was not impaired when iron or all metals were present.

The results also indicate that all metals in combination may have a stronger effect on colour removal inhibition than can be explained by iron alone. Although these results were not tested further to identify which other metals have interactions with iron that further reduce decolorization, copper may be a likely suspect. In both tests, copper had slightly reduced colour removals (less than 60% reduction) compared to the control and the chromium and nickel samples (all greater than 60% colour reduction). Furthermore, adding iron and all the metals to both growth and decolorization stages had a greater impact on decolorization inhibition than adding them into the effluent alone. This may indicate either that the metabolism necessary for decolorization was not developed during the growth stage or that the fungus stored these metals creating a cumulative effect on decolorization when metals were present.

Although Fe(III) has low aqueous solubility except at pH less than approximately 4.5, it is the only stable oxidation state in oxygen containing waters (Sawyer and McCarty 1978). Even with Fe(II) added to pulp mill wastewater, Fe(III) is the expected dominant form. No precipitation of iron hydroxide was visible in the control sample run both with deionized water and ozonated pulp mill effluent, indicating the iron was in solution.

Peroxidase enzymes, including LiP and MnP, produced by *P. chrysosporium* are heme or iron based enzymes. Barr and Aust (1994) identified the peroxidase catalytic cycle, given in Figure 5.2, that heme peroxidases use to create free radicals necessary for chemical oxidation. Peroxides are important in oxidizing non-phenolic aromatics and creating

reduction mechanisms. A pathway , shown in Figure 5.3, specifically for production of peroxide radical by LiP was also proposed where the LiP reduced oxygen to an $O_2^{\bullet-}$ radical which is responsible for reducing ferric iron to its ferrous form. Ferrous iron reacts with peroxide to give a peroxide radical that is capable of reducing highly oxidized compounds, including many chlorinated organics. If excess iron interferes with this pathway and prevents peroxide radicals from forming, or consumes them before the reaction can be completed, interference with decolorization and dechlorination may be the end result.

Figure 5.2. Peroxidase catalytic cycle. Conversion of RH to R• represents oxidation of a compound by one electron to the radical form. Adapted from Barr and Aust (1994).

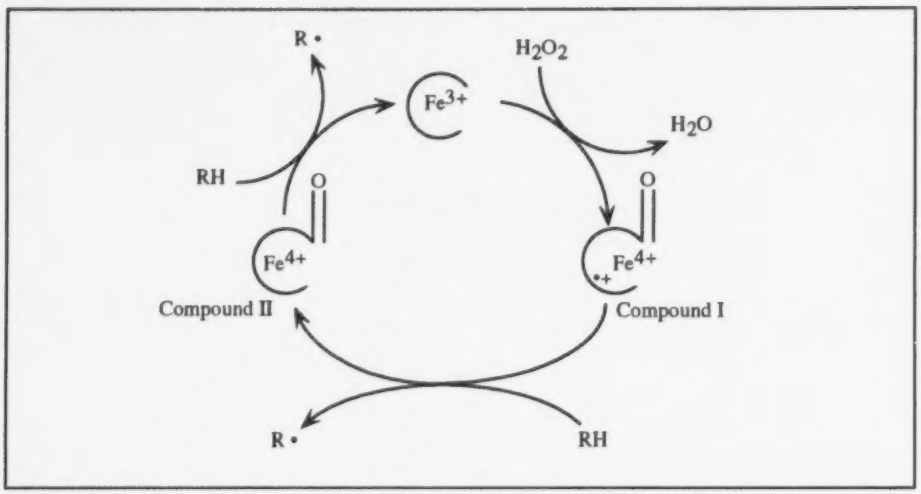
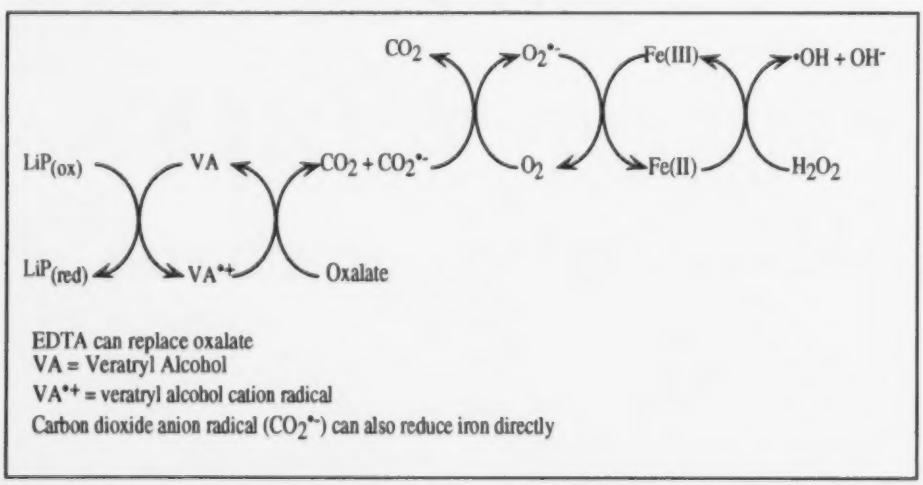


Figure 5.3. Proposed pathway for production of hydrogen peroxide radical by lignin peroxidase. Adapted from Barr and Aust (1994).



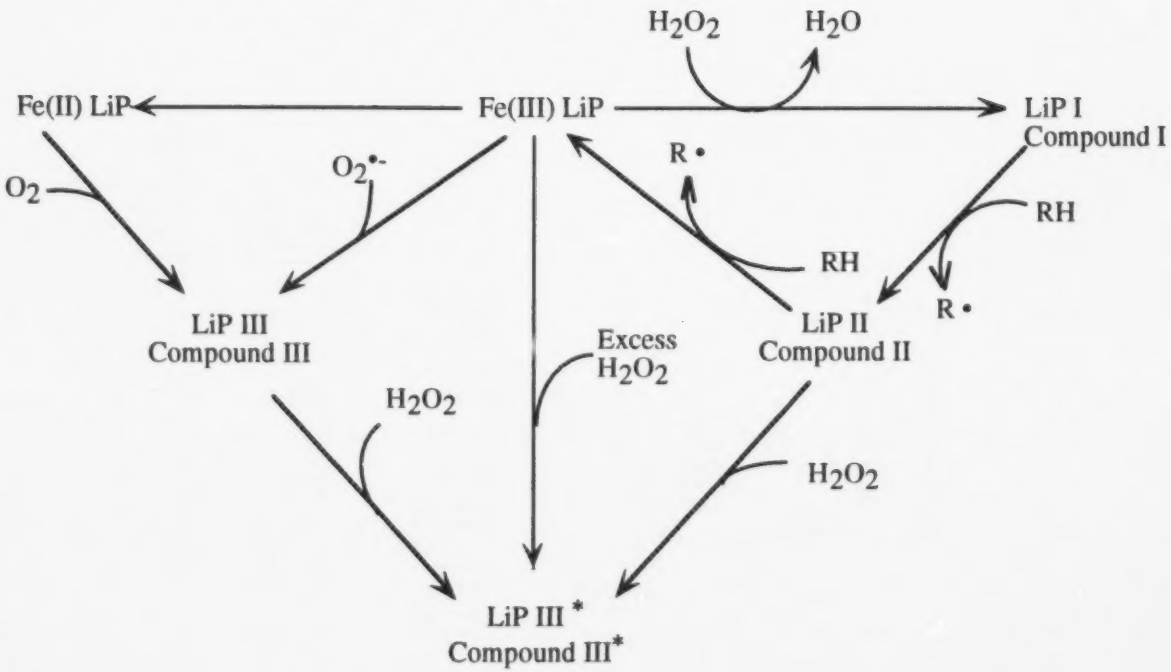


Figure 5.4. Relationships among the higher oxidation states of lignin peroxidase. Production of Compound III essentially inactivates the enzyme. R represents a compound being oxidized by one electron to its radical form R•. Adapted from Odier and Artaud (1992).

Excess H_2O_2 inactivates the LiP enzyme, thus essentially halting the lignin degradation process (Odier and Artaud 1992). A compound, called LiPIII, is formed with a small excess of H_2O_2; this compound exists as a resonance hybrid between the two iron complexes, as shown below (Odier and Artaud 1992):
$[Fe(II) — O_2] \Leftrightarrow [Fe(III) — O_2^{\bullet -}]$

Excess iron may drive the LiP oxidation state towards the LiPIII compound and inactivate the enzyme system. Relationships among the higher oxidation states of LiP are shown in Figure 5.4. Once LiPIII is formed, the catalytic cycle is terminated, whereas with the other two states, a continuous succession of electron reductions and oxidations takes place.

Iron may also form equilibrium compounds in the pulp mill effluent and nutrient solution that are somewhat toxic to the fungus. Although no visible signs of reduced growth were noted with the iron or all metals spiked samples, these results were not quantified in terms of dry weight biomass for comparison purposes. Heavy metals are known to be toxic to many living organisms (Nandan and Raisuddin 1992), but typically the more toxic metals are the ones that had no effect on fungal decolorization, namely nickel, copper, and cadmium.

Whatever the mechanism, metals, especially iron, reduce *P. chrysosporium*'s ability to remove colour from ozonated pulp mill effluent. Iron may be the principal cause for the poor performance of the bioreactors. However, this cannot be conclusively stated without further work. Ozonated pulp mill effluent samples from the bioreactor were not analyzed for metals and the metal analysis did not include all possible contaminants leaching from either the welded surface or the body of the reactors. A parameter not tested for, perhaps in the welding rods or air lines, may be acting in conjunction with iron or having an even greater effect on decolorization by *P. chrysosporium* in the bioreactor systems. As raw effluent was not tested either in the bioreactors or in the metal spiked batch tests, no direct conclusions can be drawn about effects of metal on decolorization of raw ASB effluent although similar results are expected. More testing is required to discover which metals may act in combination and to determine the lowest concentration of metals that affect colour removal.

The new stainless steel bioreactors had many advantages over the original Plexiglas design, including ease of setup, better control of temperature, aeration, pH, and evaporation, limited sludge buildup, and improved hydraulics (no short circuiting). However, all these

developments may be negated by the reactor material selection. Previous bench scale RBC or aeration fungal bioreactors all use Plexiglas (Campbell 1982; Pellinen 1988; Yin *et al.* 1989a; Prouty 1990). Batch studies typically use glass flasks for decolorization (Fukui *et al.* 1992) and lignin degradation studies (Kirk *et al.* 1978). Both of these testing procedures have limited possibility for metal contamination and would not have been a factor considered for additional evaluation.

An RBC design still may be the preferred reactor choice as numerous studies using fungi have shown (Campbell *et al.* 1982; Eaton *et al.* 1982; Prasad and Joyce 1991). Yet selection of base material for RBC construction may be a more important parameter than suspected, as these experiments have indicated. Metal sensitivity may be a limiting factor for the practicality of the combined/ozonation fungal process. The ideal conditions for biodegradation by *P. chrysosporium*, high temperature, low pH, and high oxygen concentration, combined with a high chloride and sulphate content in the pulp mill effluent, are also very corrosive even to high grade stainless steel. Stainless steel 304 grade is listed as "Not Resistant" to a 1% or less solution of HCl at 20°C with even lower resistance at higher temperatures (Atlas Alloys 1996). Although the acid concentration of HCl was not 1% in the bioreactors, *P. chrysosporium* also produced organic acids which may have had a combined effect with added HCl to increase corrosion or metal leaching in the bioreactors. Effluent from a pulp mill would also have to be evaluated for potentially high metal levels. Shock loadings are another consideration for metal inhibiting fungal decolorization especially during shutdown when cleaning or repairing equipment may increase metal loads suddenly for a short period.

5.2 Batch test results

5.2.1 Carbon and nutrient requirements

Without an easily biodegradable additional carbon source present, the colour increased in most of the fungal treated ozonated ASB effluent samples. With 1% glucose available for co-metabolism of the long chain colour compounds, greater than 50% decolorization occurred over eight days with all three strains of *P. chrysosporium* in the attached growth studies. This supports numerous other studies where colour reduction was only found to occur with a simple carbon substrate available for the fungus (Kirk *et al.* 1978; Ulmer *et al.* 1983; Joyce *et al.* 1984; Pellinen *et al.* 1988; Yin *et al.* 1989b).

However, one strain, UAMH 3642, reduced the ozonated effluent colour by approximately 30% on average over eight days without additional glucose present. This removal efficiency was lower than the 46% colour removal achieved in the samples containing glucose and nutrients in the first two attached growth batch tests. In the raw ASB samples, only 14% of the initial colour was removed by the same strain without glucose present. These results seem to support the hypothesis that ozonation creates biodegradable fragments that the fungus can use in decolorizing pulp mill effluent. The other two strains of *P. chrysosporium* tested did not show similar removals. Combinations of ozonation and biological treatments have been tested before with fungal and bacterial systems. While both processes found that ozonation enhanced treatment of pulp mill effluents, the fungal treatment with *T. versicolor* still required 20 mM of glucose for colour and chlorinated organics destruction (Roy-Arcand and Archibald 1991). The bacterial system did not focus on colour removal as one of its test parameters (Heinzle *et al.* 1992), but COD, TOC, and AOX removal efficiency improved with integrated ozonation-biotreatment.

Both Roy-Arcand *et al.* (1991) and Heinzle *et al.* (1992) used alkaline extraction stage or chlorine bleaching stage effluents rather than the biologically treated combined effluents used in the current study. Biodegradability typically only increases when ozone is applied to previously biologically treated effluents (Mohammed and Smith 1992; Mao and Smith 1995; Roy-Arcand and Archibald 1995). BOD increases were not measured in the two combined ozonation-biological procedures, but BOD can be expected to decrease with ozonation of E_1 stage effluent. This difference may have necessitated glucose addition even though some effluent organics became more bioavailable to the fungus after ozonation.

In all batch tests, transfer of fungus from the nutrient solution to the wastewater was required. Small volumes of colourless solution could not be completely drained from the fungus and were added to the wastewater, thus reducing the initial colour. Raw ASB effluent wastewater colours did not change significantly from this point even after eight days incubation with the fungus. With strains ATCC 24725 and UAMH 4521, no colour change was also found with ozonated samples not containing glucose. Only when glucose was present did colour reduction occur in these two strains.

A glucose concentration of between 1.05 and 2.1 g/L has been identified as the minimum requirements for decolorization of pulp mill effluents by *P. chrysosporium* to occur (Pellinen *et al.* 1988a; Yin *et al.* 1989b); however, relationships between fungal biomass

and wastewater volume was not considered. Once a minimum quantity of glucose was available, the same colour removal rate was achieved in a one day batch RBC MyCoR reactor. Using ozonated effluent, no pattern was discernible in regards to glucose concentrations and colour removal. With ATCC 24725, significant colour removal only occurred at 10 g/L glucose, the highest level tested. With UAMH 3642, similar removals were found at 0 and 4 g/L but the highest was still at 10 g/L. UAMH 4521 achieved its best decolorization at 4 g/L. These highly variable results were typical of the biological system especially when it was tested in ozonated ASB wastewater. The minimum glucose concentration necessary may be 4 g/L for both UAMH 4521 and UAMH 3642 and for possibly all three strains, given similarities usually noted between UAMH 4521 and ATCC 24725.

Glucose use was monitored in ATCC 24725 and UAMH 3642 cultures starting with initial concentrations of 2, 4 and 6 g/L glucose. Contrary to Yin *et al.* (1989b), greater than 30% of the initial glucose was consumed by UAMH 3642. At 2, 4, and 6 g/L, 83%, 48% and 34% respectively of the original amounts were consumed over 8 days. However, Yin *et al.* (1989b) used a one day batch RBC while this work used an eight day batch test. UAMH 3642 showed an increasing glucose depletion with higher concentrations of glucose. Conversely, ATCC 24725 consumed approximately equal quantities of glucose at each concentration tested. The two strains used glucose at different rates, although the poor colour removal by ATCC 24725 may be a factor in the lower glucose depletion. Glucose use did not appear to be proportional to colour reduction in the samples. Even when colour increased in the ozonated samples, glucose still continued to be used. As the wastewater was not sterilized before use, an unquantified amount of glucose could have been used by other microorganisms in the effluent.

Moreover, Yin *et al.* (1989b) reported that low levels of glucose are necessary for decolorization activity and that higher levels may suppress the polysaccharide degrading enzymes necessary for colour reduction. This result contradicts not only the current research, where greater than 50% colour was removed with 10 g/L glucose present, but also previous work with *P. chrysosporium* that used the same levels of glucose and achieved high colour removals (Eaton *et al.* 1980; Campbell *et al.* 1982).

Vitamins and minerals were added to the wastewater in the first three batch tests. When these results are compared to attached growth tests #3 through #6, no significant differences compared to the error exist. Vitamins and minerals may have improved

decolorization slightly, but not enough to justify the added expense of continuing to include them during the colour removal stage. Vasudevan *et al.* (1986) found that adding trace minerals during both growth and decolorization stages, or just during the decolorization stage had similar effects of increasing average colour removal in a one day batch MyCoR reactor. The highest total number of colour units removed over the 15 day cycles occurred when only nitrogen was added either in growth or decolorization. Vitamins and minerals are necessary for lignin degradation (Kirk *et al.* 1978) especially after fungal cell reserves have been depleted. In the current research, batch tests were conducted for a maximum of ten days without continuous exposure of the fungus to fresh effluent as in the method employed by Vasudevan *et al.* (1986). The fungus may not have depleted its reserves of minerals because lower coloured effluent, long retention times, and a single effluent sample all together made conditions less stressful. Furthermore, ASB effluent is enriched with nutrients to aid in biological treatment. Small amounts of these minerals may have remained in the samples used during these tests.

Nitrogen limiting conditions were present during the batch tests as shown in the nitrogen use test when ammonium chloride was added to the wastewater. Greater than 97% of the nitrogen was consumed within the first two days in all samples. *P. chrysosporium* in raw ASB effluent did not consume nitrogen as quickly as in the ozonated samples even though 1) colour removal occurred at a faster rate in the raw samples and 2) the ozonated effluent had a higher initial concentration of NH_3-Nitrogen, most likely due to measurement errors when the ammonium chloride salt was added. After eight days, all ozonated samples were below the detectable limit of 0.18 µg/L while the raw samples still had small, but detectable amounts present. Nitrogen addition did not have a negative effect on colour reduction as all the strains of fungus removed between 57 to 65% of the initial raw ASB effluent colour and between 43 and 51% of the ozonated ASB effluent colour. Other research has stated that nitrogen addition may delay onset of lignin degradation (Keyser *et al.* 1978; Faison and Kirk 1980) or repress decolorization (Eaton *et al.* 1980). Nitrogen and minerals together do not have an additive effect (Vasudevan *et al.* 1986) in improving decolorization, but the trace metals and nutrient were theorized to activate specific enzymes essential for growth, metabolism and decolorization.

5.2.2 Carbon remaining in effluent

High concentrations of biodegradable organic matter remaining in treated effluent are undesirable because the wastewater cannot be discharged until the oxygen consuming

material is removed. An ideal system would require only enough supplemental carbon for the fungus to degrade the complex organics with no residual left in the effluent. Despite reports that *P. chrysosporium* can degrade chlorinated organics completely to CO_2, H_2O, and inorganic chlorides (Pellinen *et al.* 1988a), most research has found that some organic products of decolorization and dechlorination will remain in the treated effluent (Huynh *et al.* 1985).

In this work, TOC did not change significantly after 8 days incubation despite colour reductions greater than 50% in the suspended growth test. This may indicate that neither the glucose nor the colour causing compounds are being completely oxidized to CO_2 by *P. chrysosporium* and that byproducts of decolorization are accumulating in the treated effluent. The fungus must not have stored large quantities of carbon as new biomass since TOC of the liquid effluent did not decrease. Glucose was consumed during decolorization as previous results showed, but the end products of its use are uncertain. Given the high initial TOC values from the buffer and the glucose, significant errors could exist with these measurements.

Production of low molecular weight by products during decolorization has been supported with several molecular size distribution studies (Sundman *et al.* 1981; Fukui *et al.* 1992; Mao 1996). Whether *P. chrysosporium* shows degradation specificity for different molecular weights is still questionable. Mao (1996) found that fungal treatment preferentially degraded the high molecular weight fraction, while Sundman *et al.* (1981) found no specificity for molecular weight in the degradation of visible or UV polymer chromophores by the fungus. Degradation of colour causing compounds occurred mainly in the high molecular weight fraction in contrast to the AOX degradation which was equally distributed in all fractions, with slightly higher preference for low molecular weight compounds (Fukui *et al.* 1992).

Differences in procedures and characteristics of pulp mill effluent may cause inconsistent results. Fungal degradation is a very complex process mainly because of its nonspecific nature and waste products formed during decolorization or dechlorination of pulp mill effluent are virtually impossible to identify completely for each procedure scheme. No research using *P. chrysosporium* has to date produced a system with consistently lower total COD or BOD in the treated effluent than in the initial wastewater. The additional carbon source required still remains problematic in terms of minimum requirements, total consumption, and final concentration in treated wastewater. Use of insoluble substrate that

can be settled out after decolorization has been investigated with some success. Cellulose or primary sludge could be substituted for glucose in some systems (Campbell *et al.* 1982; Eaton *et al.* 1982) with high rates of decolorization.

5.2.3 Comparison of *Phanerochaete chrysosporium* strains

In this research, three strains were evaluated: UAMH 4521, UAMH 3642, and ATCC 24725. As previously mentioned, UAMH 4521 is cross referenced with ATCC 24725 and is most likely an identical strain. UAMH 3642 shows enough morphological differences and growth patterns to be recognized as distinct. An evaluation of these strains must consider a number of factors including maximum colour removal, average colour removal, variability in results, and colour removal without an additional carbon source. Although UAMH 3642 performed most consistently in terms of average colour removal with and without nutrients, the other two strains achieved higher maximum removals. The decision was made to focus on one strain, ATCC 24725, for three reasons: 1) it was the strain used in the previous work which this project was continuing, 2) it had high maximum colour removals, and 3) it was the strain most commonly used in other research. More investigation into the capabilities of UAMH 3642 to remove colour from ozonated effluent without an additional carbon source is warranted, given the results of the first three batch tests.

Table 2.5 outlines the decolorization and dechlorination studies involving *P. chrysosporium* and lists the strain(s) of fungus used in each study. Early studies used the strain Burds ME-446, isolated from wood chips in storage for 4 months in Maine, U.S.A. and also listed as ATCC 34541. Some work was also conducted using Burds HHB-6251, which is the same strain as UAMH 3642 and is also listed as ATCC 34540. When Eaton *et al.* (1982) screened several strains for growth and decolorization, an isolate termed BKM F-1767 (ATCC 24725) was found to have better decolorization abilities than the other two strains. This strain was subsequently used almost exclusively, especially in MyCoR reactor studies. Michel *et al.* (1991) tested ME-446 and mutants derived from this strain to determine specific enzyme activities. Yin *et al.* (1989b) also listed ME-446 as the isolate used although a paper published the same year by the same authors gave the strain used as BKM F-1767. Bergbauer and Eggert (1992) termed a strain of *P. chrysosporium* as K-3, an identification which has no cross references to known strains in culture collections.

ATCC 24725 (BKM F-1767) has been widely used with notable success in decolorizing and dechlorinating pulp mill effluent. This research uses the same strain in many of the experiments for comparison with previous work. In attempting to decolorize combined pulp mill effluent without an additional carbon source, other strains or mutants of these strains may be more appropriate for these specific applications. Although the fungi are all *P. chrysosporium* strains, distinctions may exist, especially in the enzyme production or carbon metabolism, that would make one strain preferable over the others.

5.2.4 AOX and colour reduction relationships

AOX was determined in one set of batch studies to compare colour reductions with AOX removals. AOX and colour have been shown to be related because the chlorinated organics comprising AOX are often the same molecules responsible for colour in pulp mill effluent. However, biological processes may not degrade both equally. In a study of dechlorination of bleach plant effluent by *P. chrysosporium* (Fukui *et al.* 1992) the researchers found that the fungal treatment was more effective at removing colour in the high molecular weight fraction (>10 000) while AOX removal was greatest in the low molecular weight fraction (<1000) although some reduction occurred in all fractions. These results indicate different mechanisms may be occurring that selectively target different fractions of the effluent. Interestingly, in the total, unfractionated effluent, the same patterns were not apparent either because new medium and low weight fractions were created or because the fungus attacked the combined fractions differently.

The results obtained in this research are similar to those of Pellinen *et al.* (1988b) where a correlation was found between colour and AOX removals. Total organic chlorine and colour reduction were found to function in parallel, suggesting a metabolic connection, with higher rates of change in colour compared to organic chlorines.

Even when colour removal (based on the initial colours) did not occur, some AOX was degraded, approximately 7% in the raw samples and 21% in the ozonated samples. Adsorption of inorganic chlorine onto Plexiglas has been discovered before (Pellinen *et al.* 1988b), but this was not likely with chlorinated organics and glass flasks. Mycelial adsorption of chlorinated compounds has not been found to be a large factor in reducing AOX of pulp mill effluent.

These results show that for a given colour reduction, a corresponding AOX change may be predicted. Moreover, AOX removal percentages are similar in raw and ozonated samples, indicating that ozonation did not make high molecular weight chlorinated organics more easily biodegradable to the fungus. Using *T. versicolor*, Roy-Arcand and Archibald (1991) also found that ozone attacked AOX compounds that were not susceptible to fungal biodegradation and that ozone and biological treatment may have additive effects, rather than synergistic, on high molecular weight AOX.

Another feature of the AOX and colour correlation is that two strains of *P. chrysosporium* were included in the analysis. No difference in the colour and AOX relationship was found between the two strains and both were used to calculate a single regression equation. Further tests would be required to establish whether the relationship holds under a wider range of colour removals and experimental conditions.

5.2.5 Effect of varying growth periods

Two tests were done to evaluate growth patterns of the *P. chrysosporium* in nutrient solution and to determine an optimum growth period before decolorization. Development of an active ligninolytic enzyme system has been reported to occur within two to four days after an initial growth period (Keyser *et al.* 1978; Kirk *et al.* 1978; Eaton *et al.* 1980; Eaton *et al.* 1982; Ulmer *et al.* 1983) in both small scale batch and RBC systems. This research tested growth periods from two to nine days in relation to decolorization abilities.

During the growth test, fungal biomass, as measured in dry weight, showed little increase until day six, when it began to increase rapidly. Similar growth stages were found where stationary phases were interrupted by new growth on a 12 to 15 day cycle (Ulmer *et al.* 1983). Culture conditions differed slightly from this research, which could have accounted for distinct growth patterns.

When cultures were transferred to wastewater for decolorization, similar rates of total colour removal were achieved when the fungus was two to six days old. The average colour removal for cultures seven and eight days old achieved approximately 15% less final colour removals than the previous five samples or the last, nine day old culture. This drop in colour removal corresponds to when the culture grown in nutrient solution began to rapidly increase in mass, possibly signaling a new growth phase. If the cultures had been transferred while the fungus was in a transitionary stage, colour reduction could have been

affected. Similar results were found with lignin degradation where lignin degradation slowed while a new mycelial mat was created (Ulmer *et al.* 1983). Old cells continued to degrade lignin, but since the entire biomass was not devoted to that activity, lower rates were achieved in the relatively non-ligninolytic system.

While fungal mass showed an increase in day seven and eight when grown in nutrient solution, the final weight after decolorization was lower in the cultures started on these two days than in the previous three samples. Lower biomass should not have had that significant an effect on colour removals given the final decolorization and low fungal mass exhibited by two and three day old cultures. Perhaps introducing the cultures into ozonated pulp mill effluent during a second growth phase inhibited growth due to lack of nutrients or some toxicity. More extensive testing would be required to evaluate this possibility.

Figure 5.5 gives a comparison of fungal decolorization rates, rather than just overall removals. Percentage colour remaining was plotted against time for five of the cultures: 2, 4, 6, 8, and 9 day old cultures before transfer to wastewater for decolorization. Upon transfer to ozonated wastewater, the two day old culture initially increased the colour of the effluent then began to rapidly reduce the colour. Colour increases were not seen in any of the other ages of cultures. After the two day old culture, the curves for the four, six and eight day old fungal growth become successively flatter, demonstrating a slightly lower rate of colour removal. These three curves also show evidence of leveling off near the end of the eight day decolorization period. Nutrients may have been exhausted by this time or the fungal biomass could be shifting to another growth phase. Another possibility is that waste products accumulated during the growth period were retained in the mycelial mat during transfer and continued to build up. The nine day culture time curve is also given to show that the decolorization rate began to improve, achieving close to the same rate seen after two or four days culture time. This again supports the theory that a transitionary growth phase may have been taking place after seven or eight days of culture time.

Another aspect not seen with the summary graph given in the results section (Figure 4.9) is the variability of replicates. Cultures grown for two to six days, and nine days before transfer to ozonated wastewater showed much less variability in the replicate samples than did the ones transferred after seven and eight days which differed in final colour removals by 38 to 59%, as given in Appendix D. The low colour removals seen on days seven and eight may be a product of experimental error and not a true indication of fungal growth changes at all. However, when good replication was achieved on all but those two sets of

samples, random error may not be the cause for variation. Inconsistencies may result from changes that the fungal biomass is undergoing in transition to a different growth stage. If this was possible, length of culture time could be considered as a means to reduce variability in decolorization by *P. chrysosporium*.

These results indicate that long growth periods are not necessary for decolorization by *P. chrysosporium*. Even with low biomass concentrations, two day old cultures were able to decolorize ozonated effluent by 64% over eight days. Longer periods may be detrimental to colour removal performance because the ligninolytic enzyme system may not function as efficiently during transitionary phases of growth.

Spore age may be another factor that influences growth and decolorization. Most research work uses a spore innoculum to start cultures (Kirk *et al.* 1978); yet no indication of the age of the spores is usually given. The spore innoculum is based on an absorbance that gives an approximate spore concentration. However, this method does not ensure spore viability. The current research found that storage humidity, fungus strain, and age of the spore plates greatly influenced growth and decolorization. Attempts were made to standardize the inoculation method to achieve consistent results. Most consistent results were obtained when one week old plates were used to start cultures. Fewer spores had been produced after one week compared to three week old plates, but actively growing mycelium was still present on the plates. This allowed shorter culture times (four days compared to seven) to achieve enough biomass for the colour removal experiments. Older plates may have suffered from lack of spore viability and high quantities of dead biomass interfering with the spore concentration measurement.

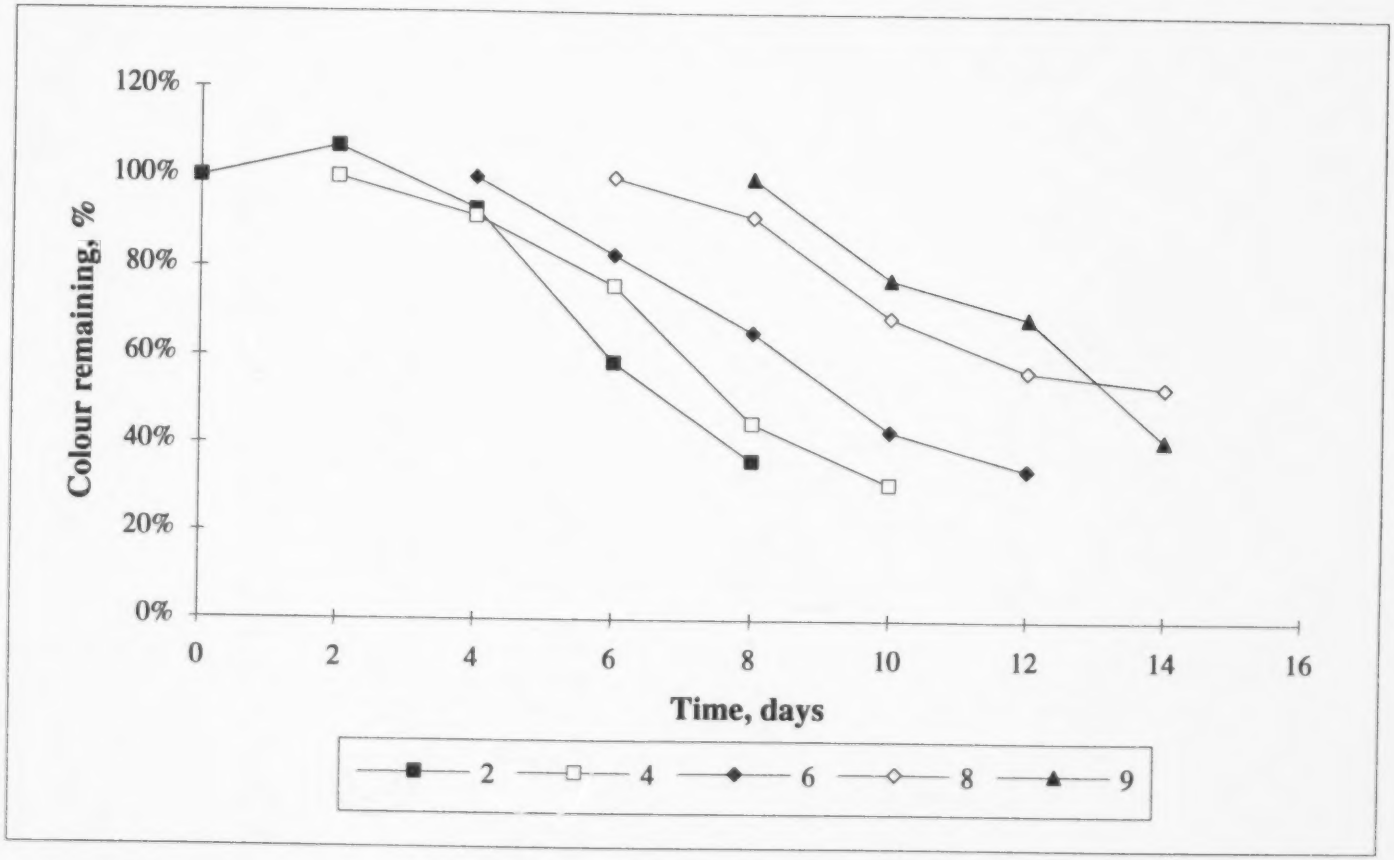


Figure 5.5. Decolorization with varying lengths of initial culture time. Results are averages from duplicates in the growth and decolorization test. Growth times were 2, 4, 6, 8, and 9 days in nutrient solution.

5.2.6 Initial colour concentration

In batch tests with a supplemental carbon source, the fungus *P. chrysosporium* typically decolorized raw ASB wastewater at twice the rate, in terms of colour units per day, as it decolorized the ozonated ASB wastewater. Moreover, kinetic studies of colour removal have found higher removal rates at higher colour concentrations (Campbell *et al.* 1982; Yin *et al.* 1989a; Yin *et al.* 1989b; Prouty 1990). ASB effluent was concentrated and ozonated to determine the effect of initial colour concentration on decolorization rate.

The two main considerations in this test were to determine if 1) a higher initial colour would increase the colour removal rate and 2) ozonating the concentrated effluent would increase the BOD to support fungal colour removal without an extra carbon source. A first test with 10 g/L glucose was run to evaluate the first point under ideal conditions. Results from this test showed poor colour removals, ranging from 14 to 30% in the concentrated raw ASB effluent and from 11 to 22% in the ozonated effluent, with initial colours of 2360 and 1580 CU, respectively. A second test without glucose was not run because of the limited colour removals even with an additional carbon source and because limited concentrated sample was available. Even with an increased BOD of 52 mg/L in the ozonated concentrated sample and additional easily biodegradable carbon added as glucose, significant colour removals did not occur.

One possible explanation for these results is the method of concentration used. Freeze drying concentrates everything equally, which means salts and compounds with potential toxicity were also approximately doubled in concentration. These may have contributed to inhibition of the fungal decolorization process. Other studies usually start with alkaline extraction effluent, a more concentrated colour source than combined effluent. To concentrate this source further, ultrafiltration has been used (Yin *et al.* 1989a) with a molecular weight cutoff of 5000. This method would not only exclude most salts and chlorides, but would also remove much of the low molecular weight toxicity (<1000), which contributes a major portion of first alkaline extraction stage toxicity (Fukui *et al.* 1992).

Reaction kinetics will determine decolorization rates and necessary reactor retention times. *P. chrysosporium* decolorization kinetics have been described and are represented in Figure 5.6 (Yin *et al.* 1989a). Improvements to the MyCoR RBC reactor have led to colour removal being most rapid within the first hour (Yin *et al.* 1989a) in contrast to the original studies where a six to seven hour lag time occurred with initial contact between the fungus

and the wastewater (Campbell *et al.* 1982). In batch tests, a zero order reaction follows the rapid colour reduction until an inflection point around 8000 to 8500 CU where the reaction rate switches to first order kinetics. These stages are explained assuming that destruction of chromophores in the effluent takes place rather than degradation of chlorolignin structures.

Yin *et al.* (1989a) developed equations to predict colour removal based on the initial colour concentration and experimentally determined reaction rate constant. These equations cannot accurately predict colour removal obtained in the current research batch studies. When detention time was increased to two days, the first order reaction rate decreased from 0.047 to 0.014 (Yin *et al.* 1989a). These results may indicate that at even longer retention times, such as the seven or eight days used in this study, and at lower initial colour concentrations the established relationship would not hold. Ozonated effluent, with an even lower initial colour, would be expected to have an even lower reaction rate constant based on this prediction approach.

A trade off obviously exists with potential use of the combined ozonation/biological decolorization system. Higher colour concentrations improve decolorization rates and the highest coloured effluent is from the alkaline extraction stage stream. However, BOD and colour in this effluent both decrease with ozonation, reducing available easily biodegradable carbon and slowing down fungal decolorization kinetics. Combined, biologically pretreated pulp mill wastewater would increase in BOD, but decrease in colour concentration with ozone application. Even if sufficient BOD was available for the *P. chrysosporium* to use in colour degradation, long detention times would be required because of the decreased colour removal rate.

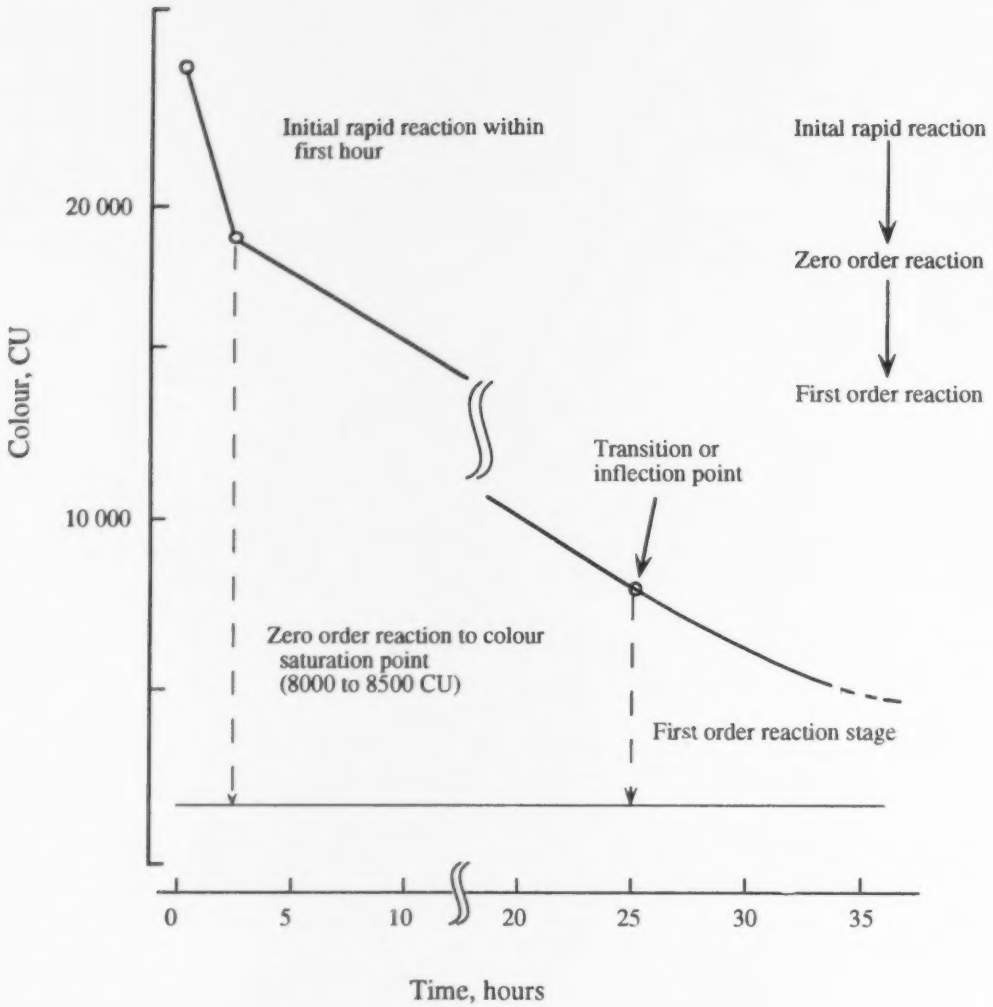


Figure 5.6. Typical kinetics for fungal colour removal from bleach plant effluent in an RBC. Reaction rate changes over course of colour removal, dependent on colour remaining in the effluent. Adapted from Yin *et al.* (1989a).

5.2.7 Improved medium evaluation

An "improved medium" was described as a mineral and nutrient supplement to improve decolorization in ozonated pulp mill wastewater (Mao 1996) without an additional carbon source. Using 10 ± 0.5 g (wet weight) of fungus in 150 mL of ozonated wastewater, approximately 45% of the original ozonated colour (≈850 CU) was removed after one day incubation in the previous study. Attempts to duplicate this work did not achieve similar removals. In ozonated wastewater, an average of 33% of the initial 460 CU was removed after seven days incubation. Surprisingly, the same experimental procedure using raw ASB effluent with the improved medium yielded 47% decolorization after seven days with an initial colour of 1220 CU. These findings demonstrate higher total colour removals in raw ASB wastewater (570 CU) compared to ozonated ASB wastewater (150 CU) with only the addition of improved medium and no additional glucose. In no previous batch tests had colour removal occurred in raw ASB wastewater without an additional carbon source present. Low BOD concentrations were thought to make decolorization in this wastewater impossible without glucose augmentation.

Since comparative studies showed that smaller quantities of fungal biomass grown on foam cubes removed more colour than the high concentration of suspended biomass used previously, attached growth studies were used to further evaluate the improved medium.

The initial batch tests contained the same mineral supplement as the improved medium, but had shown no decolorization without glucose present. Comparing the composition of the improved medium to factors that had been investigated in batch tests, three variables were identified as potential factors that could have caused colour removal with improved medium. Tween 80, $MnSO_4$, and buffer were chosen for further testing. Buffer had been added during the initial batch tests, but was included again to evaluate different concentrations and possible interactions with the other two factors.

The first factorial test using ozonated wastewater had a high standard error which essentially masked most effects or interactions. The second tests used raw ASB effluent and an attached growth test method. Centre points, using standard improved medium concentrations, achieved 50% colour reduction in three days and 59% after seven days incubation starting from an initial colour of 1150 CU.

The mean colour removal was 67% after seven days which is equivalent to a decrease of approximately 770 CU. Increasing the Tween 80 concentration improved colour removal

slightly, by 4%, which is not great enough to justify the use of this expensive chemical at the highest level of addition (0.9 mL/L). Manganese addition had a similar effect, improving decolorization by 5%. Buffer alone had the largest single effect, decreasing the amount of colour reduction by 24% or 270 CU. This result was surprising as the buffer was assumed to have a negligible role in the improved medium. The only interaction of significance compared to the standard errors was between the buffer and manganese. When manganese and the buffer were both added at the high level, colour reduction increased by approximately 6% or 70 CU.

Tween 80 has been shown to have beneficial effects on pulp mill effluent decolorization by *P. chrysosporium* (Vasudevan *et al.* 1986) and to improve enzyme activity (Asther *et al.* 1987; Kaya *et al.* 1995). Tween 80 added in a concentration of 0.3% was hypothesized to favor a greater enzyme release from the fungal cell than would normally be achieved (Vasudevan *et al.* 1986) as well as acting synergistically with nitrogen and mineral addition. This detergent may enhance enzyme transport through modification of the plasma cell membrane, but it has also been shown to supply the fungus with saturated and unsaturated fatty acids which it depletes from solution (Asther *et al.* 1987). Whether or not Tween 80 can supply *P. chrysosporium* with sufficient carbon to degrade colour causing compounds has not been investigated. Tween 80 may be a rich supply of carbon, if the fungus can access it, because the theoretical COD is greater than 2000 mg/L/g Tween 80.

Manganese is likely a key component of the ligninolytic enzyme system of *P. chrysosporium* because of its association with manganese peroxidases (Michel *et al.* 1991). While high levels of manganese stimulated MnP production and enhanced decolorization in one study (Michel *et al.* 1991), lignin degradation was inhibited in earlier research (Jeffries *et al.* 1981). Again, interactive effects were found among manganese and other minerals.

Phthalate has been used as a buffer in other research (Kirk 1978; Reid 1979) because it cannot serve as a carbon source for the fungus. However, *o*-phthalate may inhibit ligninolytic activity at higher concentrations (Reid 1979) and make it an unsuitable pH control. This was confirmed in the improved medium evaluation.

During the improved medium evaluation, visual observations noted the fungal biomass assuming a darker colour around the edges, in almost a burnt appearance. As adsorption is not assumed to be a colour removal mechanism, the darker biomass was puzzling. In desorption experiments conducted at the end of the improved medium test, colour was

released from the fungal biomass attached to foam sponges. High suspended solids were present in the desorption samples, which may have contributed to increased colour. Nevertheless, if adsorption did not take place, any fungus suspended in the liquid should still be creamy white coloured. Tween 80, because of its viscous properties, may have increased adsorption tendencies. This hypothesis would require further testing and a means to quantify the colour released after desorption.

Improved medium does promote decolorization without glucose addition in both raw and ozonated ASB effluent. These results are contradictory to previous studies that assumed enhanced colour removal only in ozonated wastewaters. The mechanisms or factors that cause decolorization without glucose addition are still uncertain. Tween 80 may serve as a carbon source for the fungus, improve enzyme transport, or promote adsorption of chromophores to the fungal surface. $MnSO_4$ addition may supply a limiting nutrient necessary for ligninolytic enzyme production. Phthalate buffer should not be used as a pH control as it has a strong inhibitory effect on decolorization in improved medium.

5.3 Changes in wastewater characteristics after ozonation

As ozonation breaks up long chain organics into smaller compounds, the colour of the pulp mill effluent decreases. However, some colour may return following ozonation, possibly due to further degradation, recombination of compounds, or addition of functional groups during aeration. Very little information is available in the literature on colour increases under varying conditions after ozonation. Colour return after ozonation, if significant in the actual treatment conditions, will affect the quantity of ozone required to meet the final discharge standards. In addition, the final BOD and pH may be affected depending on the retention time and temperature after ozonation.

The colour did not increase as much as expected from previous batch studies. The peak colour was generally reached within three days, then declined slightly to the final value. Evaporation was not largely responsible for colour increases as the evaporation controls showed negligible evaporation rates of about 0.6 to 1.1% at the temperatures evaluated. This test may have shown different results from the batch studies because of the pulp mill effluent sample used, which may be considered to be more dilute than the samples from before shut down of the mill occurred. If the extent of ozonation is a factor in colour increase, then the sample used in this study would be more ozonated than the earlier sample. During ozonation, the colour change occurred much more rapidly within the 30

minutes of ozonation and substantial ozone off gas was produced, indicating less of a demand for the ozone. With the prior sample, little or no off gas was detected.

The results from this test on aerating ozonated pulp mill effluent indicate that some colour return can be expected depending on the pH, temperature, and time before discharge. Ozonation may increase the biodegradability of the ASB wastewater, as shown by the rapid decrease in BOD at optimal conditions for bacterial growth. The pH of the wastewater will also increase with temperature and depending on the initial pH. This increase also occurs rapidly within the first two days. In application of ozonation for treatment of pulp mill wastes, these results should be considered in determining the ozone dose necessary to meet discharge requirements and in placement of the ozone system to obtain the maximum benefits of BOD decrease without permitting an excessive increase in colour or pH.

5.4 Limitations to the combined ozonation/biological treatment system

One of the research objectives was to investigate limitations to the combined ozonation/biological treatment system. Many reservations have been identified in previous sections, but will be summarized here again.

Colour removal occurs at a faster rate with a higher initial colour (Yin *et al.* 1989a; Prouty *et al.* 1990), encouraging most research to focus on using concentrated colour streams such as bleach plant effluent. Ozonation oxidizes colour compounds in these effluents, thus reducing colour, but does not increase biodegradable organics that could provide a cosubstrate for the fungus and eliminate glucose use. Research has established that an extra carbon source would be necessary for decolorization of bleach plant effluents with *P. chrysosporium*, increasing process costs. Combined ozonation/biological treatment only increases BOD in previously biologically treated effluent (Mao and Smith 1995; Roy-Arcand and Archibald 1995). This effluent is typically combined streams, reducing colour concentration greatly and decreasing the rate at which *P. chrysosporium* is capable of removing colour. This trade-off may limit effectiveness of the process. Highly concentrated colour streams may be rapidly and efficiently treated with *P. chrysosporium*, but high costs will be incurred for the additional nutrient requirements. With ozonated effluent, some potential still exists for colour removal without an additional carbon source; however, the long retention times necessary to achieve adequate decolorization would be prohibitive.

Research using an additional carbon source for fungal decolorization has identified final COD in treated effluent as a potential problem with the system (Pellinen *et al.* 1988; Yin *et al.* 1989b). Final COD would still be greater than 5000 mg/L in decolorized effluent using a soluble carbon source in the minimum concentration necessary for colour removal. When the effluent source is directly from the bleach plant, conventional biological treatment could still be used after decolorization to remove residual biodegradable organics before discharge. The combined ozonation/biological system is at another disadvantage because it already has been biologically treated in an ASB. To require another stabilization basin to remove organics added during fungal treatment would require high capital costs and redesign of the system.

No research has investigated microbial characteristics of the final treated effluent. Fungus carry over in effluent has been identified in this research. Either spores released during fungus growth or mycelium sloughed off in the reactors, would be discharged in the effluent. Given the vastly different conditions between usual discharge environments, i.e. neutral pH, low nutrients, low temperature, compared to optimum growth conditions for the fungus, the fungus may be killed rapidly in the receiving environment. However, the potential for the fungus to survive these adverse conditions are not known. *P. chrysosporium* is not considered a biohazard, but if it is released into a new ecosystem, it could have unexpected consequences.

To efficiently operate a fungal decolorization system, optimum conditions must be provided. For *P. chrysosporium*, these conditions include high temperature and low pH. Most conventional biological systems such as an ASB already operate in the high temperature range, but at close to neutral pH. To bring the pH down to from neutral to pH 4.5 would require an acid stream and separation of the fungal treatment from preceding treatment systems. Fortunately, most bleached kraft pulp mills would have capabilities to adjust the pH, but this may further reduce colour concentration, negatively affecting removal rates. Possible shocks to the system are another concern. Effects of sudden pH changes, in the event of a control systems failure or plant variability, temperature shifts, or variations in wastewater characteristics are largely unknown and would have to be identified further before scale up is considered.

Decolorization by *P. chrysosporium* was found to be highly sensitive to trace metal toxicity, especially from iron. This has implications in reactor material choices and characterization of individual wastewaters. The area of trace compound toxicity has not

been a focus for research but may affect the application of this process. Other inhibitors that are still unidentified may have an even greater effect on fungal decolorization.

Other fungi have become the focus of more recent work. *Trametes versicolor* may have more potential for decolorization and dechlorination of kraft bleach effluents because it is easily immobilized in polymer gels and may require less carbon addition (Pallerla and Chambers 1996). Research that completely eliminates the biological aspect has looked at enzyme initiated colour reduction, biological pulping, or biological bleaching without fungus present (Ander *et al.* 1990; Ferrer *et al.* 1991). Some preliminary success has been reported; however the complex combination of enzymes produced by most white rot fungi in lignin degradation is not completely understood and isolated enzymes such as LiP actually lead to repolymerization of lignin molecules (Odier and Artaud 1992).

Other potential drawbacks to fungal decolorization include colour removal lifetime of the fungus, regeneration or growth periods, trace nutrient requirements, air borne spore release, and biomass accumulation. These, as well as the ones previously discussed, show that although much work has already been conducted, research has yielded many more questions which still require investigation. Industry needs and research potential both need consideration when choosing a direction for further work into fungal decolorization of pulp mill effluent.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Combined ozonation/biological treatment process may not be well suited to colour removal from ASB effluent because higher colour removal rates occur with higher initial colour concentrations. However, sufficient biodegradable organic matter does not exist in raw ASB effluent to support decolorization without an additional carbon source. This trade-off may indicate that the two processes are not compatible.

Microbial competition does not seem to be a factor in colour reduction of ASB pulp mill effluent by *P. chrysosporium*. Bacteria, yeasts and other fungi were found in nutrient enriched ASB pulp mill effluent that had undergone successful decolorization by *P. chrysosporium*. Heat treatment of the wastewater was not necessary for favourable colour removal.

Iron and a combination of iron, nickel, cadmium and copper adversely affect decolorization of ozonated pulp mill effluent by *P. chrysosporium*. Iron added in the concentration of 10.5 mg/L reduced colour removals to approximately 21% compared to 78% in the control sample without metals. All metals in combination reduced the colour by only 14% indicating a possible interaction with iron and one or more other metals. The mechanism of iron interference with decolorization is unknown but may involve enzyme inactivation or toxicity.

Iron may have leached from the bioreactor body, air lines, or welds to inhibit decolorization by *P. chrysosporium*. Other research had all used Plexiglas reactors or glass containers and would not have encountered this problem. Reactor material may be a more important choice than previously considered.

Without an additional carbon source, one strain of *P. chrysosporium*, UAMH 3642, is capable of achieving over 30% decolorization after eight days incubation in ozonated ASB effluent. However, colour reduction occurred at a faster rate when glucose was present in the ozonated samples. Colour reduction was not possible with any strain using raw ASB effluent without glucose present. ATCC 24725 and UAMH 4521 did not reduce colour without glucose present in the raw and ozonated ASB wastewater. These two strains achieved higher colour removals than the UAMH 3642 when glucose was added to the ASB effluent.

High concentrations of BOD will occur in the treated effluent if glucose or another soluble carbon source is provided during fungal decolorization. Subsequent biological treatment may be necessary, adding to the expense and process requirements of the combined ozonation/biological treatment process.

Cultures of *P. chrysosporium* were able to achieve colour reductions greater than 60% when grown in nutrient solution for two to six days before transfer to pulp mill effluent. Longer times do not improve decolorization and result in greater final quantities of biomass.

Colour reduction is possible in raw and ozonated ASB effluent without glucose addition when improved medium, consisting of minerals, Tween 80, and buffer, is added to the wastewater. Raw ASB effluent colour can be reduced by 67%, or 770 CU, after seven days with the improved medium. Phthalate buffer inhibited colour reduction, while Tween 80 and manganese improved decolorization. Tween 80 may provide the carbon source necessary for the fungus.

Aeration after ozonation returns up to 30% of the colour, increases pH, and affects BOD, depending on the initial pH and temperature of the system. Colour and pH changes are most significant at higher temperatures where high temperatures and low pH cause the greatest colour increase.

6.2 Recommendations

If an additional carbon source is necessary for fungal decolorization to occur at a practical rate, the combined expense of both ozonation and fungal treatment would make this process much less feasible. Any further research should identify if practical means exist to improve fungal decolorization without an extra carbon source.

Methods to reduce variability in the biological testing should be investigated. High errors among tests and between replicates created difficulties in separating true effects from products of variability. For optimization, better statistical control of variation is needed.

More investigation into the capabilities of UAMH 3642 to remove colour from ozonated effluent without an additional carbon source is warranted. Optimization may improve the average colour removal rate above 30% decolorization in eight days. Research is warranted into the mechanisms that enable this strain of *P. chrysosporium* to remove colour from ozonated pulp mill effluent when other strains require an additonal carbon source. This study was not able to identify whether ozonation created enough biodegradable organics for this strain to use or whether ozonation reduced the complexity of the colour causing molecules enough for biodegradation to begin.

Because some metals, especially iron, may inhibit colour removal by *P. chrysosporium* , reactor material and other sources of metal contamination must be considered. Metal concentrations causing inhibition and biochemistry of the mechanisms involved should be identified for possible solutions to this problem. Bioreactor modifications to reduce metal leaching may be necessary and could involve coating metal components

Improved medium may have potential to aid in fungal colour removal, but more information is needed on whether adsorption or enzyme stimulation is the principle factor.

Concentrating effluents through membrane filtration before ozonation may still have potential to improve decolorization by increasing the removal rate and providing more available easily biodegradable carbon. Batch studies are recommended to test this possibility before a bench scale bioreactor is used.

BOD_5 tests with acclimatized bacteria are not good indicators of fungal biodegradability. A better means to assess biodegradability of pulp mill effluent, or other wastes, by fungi is needed.

Shock loadings or sudden effluent changes were not investigated in this research. These factors require investigation in terms of pH, temperature, loading, contaminants, and toxicity changes and their effect on final effluent quality.

Much of this work focused on batch studies. Bioreactor studies are needed to evaluate more realistic operating conditions and to better optimize the system.

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APPENDIX A

Monitoring Requirements for the Weyerhaeuser Canada Ltd. pulp mill in Grande Prairie, Alberta established by Alberta Environmental Protection
License No. 92-WL-234

Table A.1. Monitoring requirements for the Weyerhaeuser Canada Ltd. pulp mill in Grande Prairie, Alberta established by Alberta Environmental Protection

Water contaminants and parameters to be monitored	Frequency	Sample type	Samples
BOD_5	1/day	composite	1, 2
	1/week	grab	3
TSS	1/day	composite	1, 2
	1/week	grab	3
	1/month	grab	4
AOX	3/week	composite	1
Colour	1/day	composite	1
Resin and fatty acids	take with Acute Lethality sample	take with Acute Lethality sample	1
Bioassay (Acute tests: Rainbow trout, *daphnia magna*, and Sublethal/Chronic toxicity test)	see Section Three	grab	1
Flow	continuous	recorder	1, 2, 3[1]
	1/day	estimate	3
	1/month	estimate	4
Temperature	continuous	recorder	1
Oil and grease	1/week	grab	3
	1/month	grab	4
BOD_u	1/three months	composite	1
COD	1/day	composite	1, 3[1]
	1/day	grab	3
	1/month	grab	4
pH	continuous	recorder	1, 3[1]
	1/day	grab	3
	1/month	grab	4
Specific conductance	continuous	recorder	1[1], 3[1]
	1/day	grab	3
TON	1/month	grab	1
Heavy metals	1/six months	grab	1

Table A.1 (continued). Monitoring requirements for the Weyerhaeuser Canada Ltd. pulp mill in Grande Prairie, Alberta established by Alberta Environmental Protection

Water contaminants and parameters to be monitored	Frequency	Sample type	Samples
Manganese	1/month	grab	1
Chromium	1/month	grab	1
Chlorate/Chlorite[2]	1/week	grab	1
2,4-Dichlorophenol	1/month	grab	1
Chlorinated phenolics	1/month[3]	grab	1
Chloroform	1/month	grab	1
Total nitrogen, total phosphorus, and other nutrients	1/week	grab	1
Total phenols	1/month	grab	1
Organic priority pollutants	1/license period	grab	1
Total dissolved solids	1/month	grab	1
E. coli	1/month	grab	1
Dioxins and furans[4]	1/month[5]	composite	1
Sulphide (extractable)	1/month	grab	1

Legend for the samples

Samples	Sample location
1	The effluent prior to mixing with the Wapiti River or any other waters (including surface runoff), and is the designated sampling location for all wastewater which is discharged from the wastewater treatment facilities.
2	Influent to the aerated stabilization basin, and is the designated sampling location for all wastewater which enters the aerated stabilization basin, except leachate from Landfill A.
3	Discharge from the storm sewer, but prior to the discharge mixing with the Wapiti River, and is the designated sampling location for those flows permitted under subsection 3.17.
4	Leachate collection sump for Landfill A, and is the designated sampling location for the leachate from Landfill A, which is discharged to the aerated stabilization basin.

Notes:

1 Effective August 1, 1993; corresponding grab sample requirements are also deleted at this time.

2 If no detections after one year, sampling frequency will be reduced to 1/month.

3 Chlorinated phenolics are to be measured 1/week in February and March of each year until 1995, wherein the frequency shall revert to 1/month.

4 To be sampled, preserved, analyzed, and reported according to the Reference Methods (Section Nine) and the "Pulp and Paper Mill Effluent Chlorinated Dioxins and Furans Regulations" under the "Canadian Environmental Protection Act".

5 The sampling frequency is automatically subject to modification in accordance with the "Pulp and Paper Mill Effluent Chlorinated Dioxins and Furans Regulations" under the "Canadian Environmental Protection Act".

APPENDIX B

Data collected from Immobilized Fungi Rotating Biological Contactor

January to March 1995 uses original Plexiglas reactor

April 1995 to March 1996 uses new stainless steel bioreactors

Table B.1. Data Summary for Bioreactor Effluent Samples January 1995

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	Duration of Bioreactor Operation	BOD (mg/L)	TOC (mg/L)	COD (mg/L)	AOX (mg/L)	Colour (pH 7.6, colour units)
Dec 9/94 ASB grab effluent	NA	Dec 9, 1994	NA	RD[5]	280	605.9	9.50 to 9.71	1150
Ozonated Dec 9/94 effluent	80 to 84	Jan 12 to Jan 27, 1995	NA	RD[5]	228	496 to 520	6.70 to 6.81	393
Chemically defined medium + planktonic fungus	NA	19 Jan 1995	7 days[1]	703	5162	ND	0.07 to 0.08	59
Artificial sample to simulate HRT = 5 hr	NA	19 Jan 1995	5 hr	807	4160	ND	1.50 to 1.55	99
BR Chamber I	80 to 84	19 Jan 1995 4:30 pm	4.5 hr	765	4912	11,725	0.64	108
BR Chamber II	80 to 84	19 Jan 1995 4:30 pm	4.5 hr	381	5049	11,912	0.80	93
BR Effluent[2]	80 to 84	20 Jan 1995 4 pm	28 hr	765	1817	11,483	3.38	234
BR Effluent	80 to 84	21 Jan 1995 11:30 am	~ 24 hr	777	1126	2418	ND	176
BR Effluent	80 to 84	22 Jan 1995 10 am	~ 48 hr	183	515	1500	5.16	311
BR Effluent	80 to 84	23 Jan 1995 9 am	~ 72 hr	45	360	755.6	5.54	315
BR Effluent	80 to 84	30 Jan 1995 9 am	~ 10 d	ND[4]	*[3]	556	ND[4]	ND

BR	Bioreactor
HPC	Total Aerobic Heterotrophic Plate Count
NA	Not Applicable
ND	Not Determined
RD	To be redone due to difficulties with Winkler method for dissolved oxygen.
1	Bioreactor operating for 7 days with fungus plus chemically defined medium
2	Bioreactor had to be restarted on Jan. 20/95 due to mechanical problems with pumping influent
*3	TOC being repaired
4	Analysis still to be completed
5	Samples from the bioreactor (pH 4.5) are pH adjusted to pH 2 to precipitate proteins that interfere with spectrophotometric reading. Color at pH 2.0 is not significantly different than colour at pH 4.5

Table B.1 (continued). Data Summary for Bioreactor Effluent Samples January 1995

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	Duration of Bioreactor Operation	Colour (pH 2.0, colour units)[5]	HPC (35°C, 48 hr, cfu/mL)	HPC (20°C, 7d), cfu/mL	Mold (20°C. 7d, cfu/mL)
Dec 9/94 ASB grab effluent	NA	Dec 9, 1994	NA	ND	6.9×10^4	3.6×10^5	> 200
Ozonated Dec 9/94 effluent	80 to 84	Jan 12 to Jan 27, 1995	NA	ND	1.9×10^4	9.4×10^4	> 200
Chemically defined medium + planktonic fungus	NA	19 Jan 1995	7 days[1]	ND	ND	ND	ND
Artificial sample to simulate HRT = 5 hr	NA	19 Jan 1995	5 hr	ND	ND	ND	ND
BR Chamber I	80 to 84	19 Jan 1995 4:30 pm	4.5 hr	ND	ND	ND	ND
BR Chamber II	80 to 84	19 Jan 1995 4:30 pm	4.5 hr	ND	ND	ND	ND
BR Effluent[2]	80 to 84	20 Jan 1995 4 pm	28 hr	ND	ND	ND	ND
BR Effluent	80 to 84	21 Jan 1995 11:30 am	~ 24 hr	86	ND	ND	ND
BR Effluent	80 to 84	22 Jan 1995 10 am	~ 48 hr	108	ND	ND	ND
BR Effluent	80 to 84	23 Jan 1995 9 am	~ 72 hr	116	3×10^2	5.3×10^2	2.3×10^2
BR Effluent	80 to 84	30 Jan 1995 9 am	~ 10 d	ND	ND	ND	ND

Table B.2. Data summary for bioreactor effluent composite samples January 1995. Bioreactor started January 19, 1995 12pm with a HRT of 24 hours

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	Time of Bioreactor Operation	BOD (mg/L)	TOC (mg/L)	COD (mg/L)	AOX (mg/L)
BR Composite Effluent[2]	80 to 84	20 Jan 1995 4 pm	28 hr	771	4751	4533	3.38
BR Composite Effluent	80 to 84	21 Jan 1995 11:30 am	~ 24 hr	771	1380	3246	3.74
BR Composite Effluent	80 to 84	22 Jan 1995 10 am	~ 48 hr	660	673	1500	4.79
BR Composite Effluent	80 to 84	23 Jan 1995 9 am	~ 72 hr	54	421	905	4.76

Table B.2 (continued). Data summary for bioreactor effluent composite samples January 1995. Bioreactor started January 19, 1995 12pm with a HRT of 24 hours

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	Time of Bioreactor Operation	Colour (colour units)	HPC (35°C, 48 hr, cfu/mL)	HPC (20°C, 7d), cfu/mL	Mold (20°C. 7d, cfu/mL)
BR Composite Effluent[2]	80 to 84	20 Jan 1995 4 pm	28 hr	142	ND	ND	ND
BR Composite Effluent	80 to 84	21 Jan 1995 11:30 am	~ 24 hr	232	ND	ND	ND
BR Composite Effluent	80 to 84	22 Jan 1995 10 am	~ 48 hr	302	ND	ND	ND
BR Composite Effluent	80 to 84	23 Jan 1995 9 am	~ 72 hr	405	ND	ND	ND

Table B.3. February 1995 bioreactor data. Summary for ASB effluent samples. Bioreactor started Jan 19, 1995, 12 pm and run continuously

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	BOD (mg/L)	TOC (mg/L)	COD (mg/L)	AOX (mg/L)	Colour (pH 7.6, colour units)	HPC (35°C, 48 hr, cfu/mL)	Yeast (20°C. 7d, cfu/mL)	Mold (20°C. 7d, cfu/mL)
ASB grab effluent (2 Feb 95)	NA	2 Feb 95	*1,2*	256	680	9.64	1100	3.6×10^5	7.8×10^2	3.2×10^2
ASB ozonated effluent (IIZ95-02-06)	40	6 Feb 95	*1,2*	240	640	8.05	620	-	-	-
ASB ozonated effluent (IIZ95-02-10)	40	10 Feb 95	*1,2*	*2*	*2*	*2*	*2*	-	-	-
ASB ozonated effluent (IIZ95-02-13)	60	13 Feb 95	*1,2*	238	640	7.69	580	-	-	-
ASB ozonated effluent (IIZ95-02-15)	60	15 Feb 95	*1,2*	245	620	*2*	530	$>2 \times 10^2$	$> 2 \times 10^3$	$> 2 \times 10^3$
ASB ozonated effluent (IIZ95-02-24)	60	24 Feb 95	*1,2*	*2*	*2*	*2*	*2*	-	-	-
ASB ozonated effluent (IIZ95-03-02)	80	2 Mar 95	*1,2*	*2*	*2*	*2*	500	-	-	-
ASB ozonated effluent (IIZ95-03-09)	80	9 Mar 95	*1,2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*
ASB ozonated effluent (IIZ95-03-09*)	90	9 Mar 95	*1,2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*
ASB ozonated effluent (IIZ95-03-09-1)	100	9 Mar 95	*1,2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*
ASB ozonated effluent (IIZ95-03-10-A)	110	10 Mar 95	*1,2*	251	*2*	*2*	*2*	1.6×10^5	*2*	*2*
ASB ozonated effluent (IIZ95-03-10-B)	114	10 Mar 95	*1, 2*	245	*2*	*2*	*2*	*2*	*2*	*2*

Table B.3 (continued). February 1995 bioreactor data. Summary for ASB effluent samples. Bioreactor started Jan 19, 1995, 12 pm and run continuously

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	BOD (mg/L)	TOC (mg/L)	COD (mg/L)	AOX (mg/L)	Colour (pH 7.6, colour units)	Colour (pH 4.5, colour units)	HPC (35°C, 48 hr, cfu/mL)
BR Effluent	80	01 Feb 95 (09:00)	*1,2*	241	*2*	6.42	620	170	*ND*
BR Effluent	80	02 Feb 95 (09:00)	*1,2*	238	600	6.45	570	170	*ND*
BR Effluent	80	03 Feb 95 (09:00)	*1,2*	241	510	6.57	610	170	*ND*
BR Effluent[3]	40	07 Feb 95 (09:00)	*1,2*	606	1670	6.04	550	190	*ND*
BR Effluent	40	08 Feb 95 (09:00)	*1,2*	355	880	6.41	560	190	*ND*
BR Effluent	40	09 Feb 95 (09:00)	*1,2*	371	910	8.42	950	680	*ND*
BR Effluent[3]	40	10 Feb 95 (09:00)	*1,2*	321	810	8.58	1100	750	*ND*
BR Effluent	40	11 Feb 95 (10:00)	*1,2*	316	820	9.12	1140	920	*ND*
BR Effluent	40	12 Feb 95 (10:30)	*1,2*	297	750	8.36	780	-	*ND*
BR Effluent	60	13 Feb 95 (09:00)	*1,2*	287	730	8.82	650	-	*ND*
BR Effluent	60	14 Feb 95 (09:00)	*1,2*	280	740	8.39	630	-	*ND*
BR Effluent	60	15 Feb 95 (09:00)	*1,2*	287	720	-	600	-	2.6×10^4
BR Effluent	60	16 Feb 95 (09:00)	*1,2*	291	730	-	840	625	*ND*
BR Effluent[3]	60	17 Feb 95 (09:00)	*1,2*	307	810	-	800	630	*ND*
BR Effluent	60	23 Feb 95 (09:00)	*1,2*	309	-	-	930	620	*ND*
BR Effluent	60	24 Feb 95 (09:00)	*1,2*	292	-	8.39	850	610	*ND*
BR Effluent	60	25 Feb 95 (09:00)	*1,2*	306	845	7.94	800	-	*ND*
BR Effluent	60	26 Feb 95 (09:00)	*1,2*	286	710	8.14	870	580	*ND*
BR Effluent	60	27 Feb 95 (09:00)	*1,2*	297	730	8.43	920	640	*ND*
BR Effluent	60	28 Feb 95 (09:00)	*1,2*	315	760	8.61	1000	740	*ND*
BR Effluent	60	01 Mar 95 (09:00)	*1,2*	310	800	8.95	1020	700	*ND*
BR Effluent[3]	60	02 Mar 95 (09:00)	*1, 2*	288	760	8.70	960	800	*ND*

BR	Bioreactor
HPC	Total Aerobic Heterotrophic Plate Count
NA	Not Applicable
ND	Not Determined
1	BOD seed had to be restarted
2	Analysis not completed
3	Mineral media plus glucose added to the bioreactor, with or without ozonated effluent also being added

Table B. 4. March 1995 bioreactor data. Data summary for bioreactor samples collected March 1995

Sample type	Ozone dose mg/L	Date collected	TOC mg/L	COD mg/L	AOX mg/L	Colour (pH=4.5) CU	Colour (pH=7.6) CU
Bioreactor effluent	60	1 March 1995	310	804	8.95		
Bioreactor effluent	60	2 March 1995	288	758	8.70		
Bioreactor effluent	60	6 March 1995	1400	3409		480	580
Ozonated ASB effluent	80	1 to 16 March	243			480	500
Bioreactor effluent	80	13 March 1995		4545		ND	ND
Bioreactor effluent	80	14 March 1995		2992	5.71	350	430
Bioreactor effluent	80	15 March 1995	349	850	7.15	460	560
Bioreactor effluent [1]	80	16 March 1995	254	601	7.23	370	590
Bioreactor effluent [1]	80	17 March 1995	229	537	6.93	340	540
Bioreactor effluent [1]	80	18 March 1995	221	533	6.29	330	510
Bioreactor effluent [1]	80	19 March 1995	214	513	6.11	300	470
Bioreactor effluent [1]	80	20 March 1995	213	492	6.11	290	470
Bioreactor effluent [1]	80	21 March 1995	231	545	6.57	520	510

ND Not determined
1 Began to use sterile, deionized water as the evaporation makeup solution rather than ozonated ASB effluent

Table B.5. April 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected April 1995

Sample type	Date collected	TOC mg/L	BOD mg/L	COD mg/L	AOX mg/L	Colour (pH=4.5) CU	Colour (pH=7.6) CU
Raw effluent	29 March 1995	218	ND	587	8.60	1100	1100
Ozonated ASB effluent	18 April 1995	214	ND	515	6.03	340	360
Heated, ozonated ASB effluent (60°C, 30 min)	18 April 1995	224	ND	541	6.26	280	400
Bioreactor effluent	20 April 1995	ND	ND	15532	3.92	220	250
Bioreactor effluent	21 April 1995	2481	ND	6543	6.21	320	600
Bioreactor effluent	22 April 1995	1381	ND	3725	6.80	310	500
Bioreactor effluent	23 April 1995	715	696	1941	7.07	330	540
Bioreactor effluent	24 April 1995	385	91	968	7.15	330	410
Bioreactor effluent	25 April 1995	312	26	811	7.32	410	630
Bioreactor effluent	26 April 1995	287	18	707	7.54	400	590
Bioreactor effluent	27 April 1995	285	16	711	7.80	390	610
Bioreactor effluent	28 April 1995	282	16	704	7.73	410	630
Bioreactor effluent	29 April 1995	279	20	797	7.25	400	620
Bioreactor effluent	30 April 1995	284	18	705	7.88	410	640

Table B.5 (continued). April 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected April 1995

Sample Type	Date collected	HPC (35 °C, 48 h, cfu/mL)	HPC (20 °C, 7 d, cfu/mL)	Molds (20 °C, 7 d, cfu/mL)	Presumptive yeasts (20 °C, 7 d, cfu/mL)
Raw effluent	29 March 1995	1.1×10^6	1.8×10^6	1	1.5×10^3
Ozonated (80 mg/L) effluent	18 April 1995	1.0×10^5	4.1×10^5	2	2×10^2
Heated, ozonated effluent (80 mg/L, 60°C, inline)	18 April 1995	8.2×10^2	2.7×10^3	Confluent*	8.5×10^3
Bioreactor effluent	21 April 1995	6.5×10^5	3.5×10^6	Confluent*	6.5×10^5

HPC Heterotrophic plate count
cfu Colony forming unit
* In the samples examined, the fungal growth covered the entire plate, making it impossible to determine if growth was a single colony or multiple colonies.

Table B.6. May 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected May 1995

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	TOC (mg/L)	COD (mg/L)	AOX (mg/L)	Colour (pH, 4.5 Cu)	Colour (pH 7.6, CU)	BOD5 (mg/L)
Bioreactor Effluent	80	1-May-95 12 AM	284	707	7.712	430	630	20.6
Bioreactor Effluent	80	2-May-95 12 AM	295	672	7.800	430	640	22.8
Bioreactor Effluent	80	3-May-95 12 AM	287	670	7.944	430	640	22.8
Bioreactor Effluent	80	4-May-95 12 AM	281	672	7.768	570	680	21.0
Raw ASB Effluent	0	8-May-95 12 AM	229	660	7.960	1210	1200	20.4
Ozonated ASB Effluent	80	8-May-95 12 AM	211	591	5.545	450	460	31.7
Bioreactor Effluent	80	9-May-95 12 AM	641					741.3
Bioreactor Effluent	80	10-May-95 12 AM	262		5.345			41.3
Bioreactor Effluent	80	11-May-95 12 AM	262		4.266	460	640	15.9
Ozonated ASB Effluent	80	18-May-95 12 AM	220	516		460	460	29.3
Ozonated heated ASB Effluent	80	18-May-95 12 AM	219	498		520	510	26.7
Bioreactor #1 Effluent	80	29-May-95 9 AM	544			310	460	
Bioreactor #1 Effluent	80	30-May-95 9 PM	246			370	500	
Bioreactor #1 Effluent	80	30-May-95 9 PM	228			390	520	
Bioreactor #1 Effluent	80	31-May-95 9 AM	236			400	530	
Bioreactor #1 Effluent	80	31-May-95 9 PM	226			390	530	
Bioreactor #2 Effluent	80	30-May-95 9 AM	252			360	490	
Bioreactor #2 Effluent	80	31-May-95 9 AM	236			450	580	
Bioreactor #3 Effluent	80	30-May-95 9 AM	275			410	570	
Raw ASB Effluent	0	31-May-95 12 AM	223	560		1100	1120	15.5
Ozonated ASB Effluent	80	31-May-95 12 AM	212	496		330	340	28.0
Ozonated heated ASB Effluent	80	31-May-95 12 AM	204	462		390	400	24.9

Table B.7. June 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected June 1995

Sample Type	Ozone Dose (mg/L)	Date and Time Collected	TOC (mg/L)	COD (mg/L)	Colour (pH 4.5, CU)	Colour (pH 7.6, CU)	BOD5 (mg/L)
Bioreactor #1 Effluent	80	1-Jun-95 9 AM	220		360	510	
Bioreactor #2 Effluent	80	1-Jun-95 9 AM	253		450	610	
Bioreactor #3 Effluent	80	1-Jun-95 9 AM	247		420	580	
Bioreactor #1 Effluent	80	4-Jun-95 9 PM			250	370	
Bioreactor #1 Effluent	80	5-Jun-95 9 AM			270	410	
Bioreactor #2 Effluent	80	5-Jun-95 9 AM			250	390	
Bioreactor #1 Effluent	80	5-Jun-95 9 PM			290	420	
Bioreactor #3 Effluent	80	5-Jun-95 9 PM			280	420	
Bioreactor #1 Effluent	80	6-Jun-95 9 AM			330	440	
Bioreactor #2 Effluent	80	6-Jun-95 9 AM			360	450	
Bioreactor #1 Effluent	80	6-Jun-95 9 PM			290	430	
Ozonated ASB Effluent	80	6-Jun-95		514	440	440	31.0
Ozonated heated ASB Effluent	80	6-Jun-95	221	496	500	510	28.3
Bioreactor #1 Effluent	80	7-Jun-95 9 AM		498	300	450	7.6
Bioreactor #2 Effluent	80	7-Jun-95 9 AM		516	310	460	16.9
Bioreactor #3 Effluent	80	7-Jun-95 9 AM		534	300	460	19.1

Table B.8. November 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected November 1995

Sample	Date and Time	Colour1, CU	Colour2, CU	Colour3, CU	Comments
Initial Ozonated	IIZ951109	320	290	170	80 mg/L O3 for 30 min
Bioreactor #2 Effluent	11-Nov 9 AM	330	320	180	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	11-Nov 9 PM	330	330	180	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	12-Nov 11 AM	360	340	180	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	13-Nov 9 AM	350	340	190	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	14-Nov 9 PM	440	350	190	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	16-Nov 9 AM	350	340	190	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	17-Nov 9 AM	360	350	200	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	17-Nov 9 PM	360	370	210	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	19-Nov 9 AM	370	370	220	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	20-Nov 9 AM	360	380	210	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	20-Nov 9 PM	390	380	210	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	21-Nov 9 PM	360	360	210	36 hr td, 4 g/L glucose
Bioreactor #2 Effluent	22-Nov 9 AM	330	360	200	36 hr td, 4 g/L glucose
Initial Ozonated	IIZ951128	280			80 mg/L O3 for 30 min
Bioreactor #1 Effluent	29-Nov 9 AM	290	290	150	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	29-Nov 9 AM	260	250	120	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	30-Nov 9 AM	320	310	190	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	30-Nov 9 AM	260	250	140	72 hr td, 4 g/L glucose

Table B.9. December 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected December 1995

Sample	Date and Time	Colour1, CU	Colour2, CU	Colour3, CU	Comments
Bioreactor #1 Effluent	1-Dec 9 AM	350	330	180	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	1-Dec 9 AM	300	270	150	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	2-Dec 9 AM	340	360	210	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	2-Dec 9 AM	350	350	190	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	3-Dec 9 AM	350	360	210	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	3-Dec 9 AM	330	330	180	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	4-Dec 9 AM	370	370	210	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	4-Dec 9 AM	320	360	270	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	5-Dec 9 AM	370	390	230	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	5-Dec 9 AM	380	370	260	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	6-Dec 9 AM	380	420	210	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	6-Dec 9 AM	370	420	290	72 hr td, 4 g/L glucose
Bioreactor #1 Effluent	7-Dec 9 AM	390	380	210	66 hr td, 4 g/L glucose
Bioreactor #2 Effluent	7-Dec 9 AM	350	410	270	72 hr td, 4 g/L glucose
Raw ASB Wastewater		910			
Initial Ozonated	IIZ951207	290			80 mg/L O3 for 30 min
Bioreactor #1 Effluent	12-Dec 9 AM	240	220	120	66 hr td, 10 g/L glucose
Bioreactor #1 Effluent	13-Dec 9 AM	250	260	140	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	13-Dec 9 AM	280	270	150	72 hr td, 10 g/L glucose
Bioreactor #1 Effluent	14-Dec 9 AM	280	280	130	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	14-Dec 9 AM	270	300	150	72 hr td, 10 g/L glucose

Table B.9 (continued). December 1995 bioreactor data. Data summary for new stainless steel bioreactor samples collected December 1995

Sample	Date and Time	Colour1, CU	Colour2, CU	Colour3, CU	Comments
Bioreactor #1 Effluent	15-Dec 9 AM	300	310	150	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	15-Dec 9 AM	310	340	170	72 hr td, 10 g/L glucose
Bioreactor #1 Effluent	16-Dec 9 AM	360	360	180	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	16-Dec 9 AM	250	350	180	72 hr td, 10 g/L glucose
Bioreactor #1 Effluent	17-Dec 9 AM	280	340	170	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	17-Dec 9 AM	270	360	180	72 hr td, 10 g/L glucose
Bioreactor #1 Effluent	18-Dec 9 AM	250	320	180	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	18-Dec 9 AM	310	360	180	72 hr td, 10 g/L glucose
Bioreactor #1 Effluent	19-Dec 9 AM	310	320	170	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	19-Dec 9 AM	270	320	180	72 hr td, 10 g/L glucose
Bioreactor #1 Effluent	20-Dec 9 AM	300	310	170	66 hr td, 10 g/L glucose
Bioreactor #2 Effluent	20-Dec 9 AM	350	350	200	72 hr td, 10 g/L glucose

Colour 1 Non-centrifuged, pH adjusted to 7.6, filtered through 0.8 um filter
Colour 2 Acidified, centrifuged, pH adjusted to 7.6, filtered through 0.8 um filter
Colour 3 Centrifuged, acidified pH, filtered through 0.8 um filter

Bioreactor 1 inoculated with UAMH 4521 spores directly into reactor

Bioreactor 2 inoculated with ATCC 24725 spores on sponges outside reactor

Table B.10. January 1996 bioreactor data. Data summary for new stainless steel bioreactor samples collected January 1996

Sample	Date and Time	Colour1, CU	Colour2, CU	Comments
Initial Ozonated	IIZ960123	420		80 mg/L O3 for 30 min
Bioreactor #1 Effluent	30-Jan 9 AM	450	430	10 g/L glucose, no flow
Bioreactor #2 Effluent	30-Jan 9 AM	460	460	no glucose, no flow
Bioreactor #1 Effluent	31-Jan 9 AM	460	460	10 g/L glucose, no flow
Bioreactor #2 Effluent	31-Jan 9 AM	490	490	no glucose, no flow
Bioreactor #1 Effluent	1-Feb 9 AM	430	440	10 g/L glucose, no flow
Bioreactor #2 Effluent	1-Feb 9 AM	500	500	no glucose, no flow
Bioreactor #1 Effluent	2-Feb 9 AM	450		10 g/L glucose, no flow
Bioreactor #2 Effluent	2-Feb 9 AM	520		no glucose, no flow
Bioreactor #1 Effluent	3-Feb 9 AM	440		10 g/L glucose, no flow
Bioreactor #2 Effluent	3-Feb 9 AM	540		no glucose, no flow
Bioreactor #1 Effluent	4-Feb 9 AM	420		10 g/L glucose, no flow
Bioreactor #2 Effluent	4-Feb 9 AM	580		no glucose, no flow
Bioreactor #1 Effluent	5-Feb 9 AM	400		10 g/L glucose, no flow
Bioreactor #2 Effluent	5-Feb 9 AM	590		no glucose, no flow
Bioreactor #1 Effluent	6-Feb 9 AM	460		10 g/L glucose, no flow
Bioreactor #2 Effluent	6-Feb 9 AM	620		no glucose, no flow

Colour1 Non-centrifuged, pH adjusted to 7.6, filtered through 0.8 um filter
Colour2 Centrifuged, pH adjusted to 7.6, filtered through 0.8 um filter

Both bioreactors were inoculated with UAMH 3642 spores directly into the reactor and grown for 7 days on nutrient solution without recycle

Table B.11. February 1996 bioreactor data. Data summary for new stainless steel bioreactor samples collected February 1996

Sample	Date and time	Colour, CU	Comments
Bioreactor #1 effluent	7-Feb-9 AM	490	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	7-Feb-9 AM	630	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	8-Feb-9 AM	530	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	8-Feb-9 AM	660	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	9-Feb-9 AM	570	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	9-Feb-9 AM	670	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	10-Feb-9 AM	550	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	10-Feb-9 AM	660	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	11-Feb-9 AM	550	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	11-Feb-9 AM	720	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	12-Feb-9 AM	600	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	12-Feb-9 AM	640	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	13-Feb-9 AM	610	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	13-Feb-9 AM	610	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Bioreactor #1 effluent	14-Feb-9 AM	640	UAMH 3642, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	14-Feb-9 AM	620	UAMH 3642, ozonated ASB effluent,no glucose, no flow, no sponge rotation
Initial ASB effluent	23-Feb	1280	
Ozonated (80 mg/L) effluent	23-Feb	450	
Bioreactor #1 effluent	26-Feb-9 AM	420	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	26-Feb-9 AM	430	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	27-Feb-9 AM	450	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	27-Feb-9 AM	470	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	28-Feb-9 AM	470	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	28-Feb-9 AM	490	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	29-Feb-9 AM	520	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	29-Feb-9 AM	540	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation

Table B.12. March 1996 bioreactor data. Data summary for new stainless steel bioreactor samples collected March 1996

Sample	Date and time	Colour CU	Comments
Bioreactor #1 effluent	1-Mar-9 AM	530	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	1-Mar-9 AM	540	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	2-Mar-9 AM	490	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	2-Mar-9 AM	530	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	3-Mar-9 AM	450	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	3-Mar-9 AM	500	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	4-Mar-9 AM	420	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	4-Mar-9 AM	490	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	5-Mar-9 AM	380	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	5-Mar-9 AM	470	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	6-Mar-9 AM	420	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	6-Mar-9 AM	480	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	7-Mar-9 AM	400	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	7-Mar-9 AM	460	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	8-Mar-9 AM	430	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	8-Mar-9 AM	460	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Initial ASB effluent	11-Mar-9 AM	1240	
Ozonated (80 mg/L) effluent	11-Mar-9 AM	430	
Initial Bioreactor #1	11-Mar-9 AM	410	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Initial Bioreactor #2	11-Mar-9 AM	410	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	12-Mar-9 AM	410	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	12-Mar-9 AM	450	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	13-Mar-9 AM	430	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	13-Mar-9 AM	450	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	14-Mar-9 AM	470	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	14-Mar-9 AM	450	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation

Table B.12 (continued). March 1996 bioreactor data. Data summary for new stainless steel bioreactor samples collected March 1996

Sample	Date and time	Colour CU	Comments
Bioreactor #1 effluent	15-Mar-9 AM	490	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	15-Mar-9 AM	470	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	16-Mar-9 AM	470	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	16-Mar-9 AM	440	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	17-Mar-9 AM	490	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	17-Mar-9 AM	460	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	18-Mar-9 AM	570	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	18-Mar-9 AM	500	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #1 effluent	19-Mar-9 AM	670	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation
Bioreactor #2 effluent	19-Mar-9 AM	510	ATCC 24725, ozonated ASB effluent, 10 g/L glucose, no flow, no sponge rotation

APPENDIX C

Tracer studies from new stainless steel bioreactor to determine flow hydraulics

Table C.1. Hydraulic dye test on Bioreactor #1 using stock dye solution and rotation rate of 1 rpm.

Elapsed time minutes	Absorbency at 625 nm	
	S2	S4
0	0	0
2	0.009	0.011
4	0.013	0.011
6	0.006	0.008
8	0.006	0.007
10	0.006	0.007
12	0.007	0.01
14	0.007	0.007
16	0.009	0.007
18	0.009	0.007
20	0.007	0.008
22	0.016	0.008
24	0.02	0.009
26	0.03	0.009
28	0.039	0.008
30	0.045	0.007
32	0.064	0.009
34	0.078	0.009
36	0.093	0.01
38	0.111	0.007
40	0.123	0.009
42	0.153	0.009
44	0.183	0.009
46	0.216	0.008
48	0.239	0.008
50	0.292	0.008

Figure C.1. Bioreactor sampling locations for hydraulic dye tests

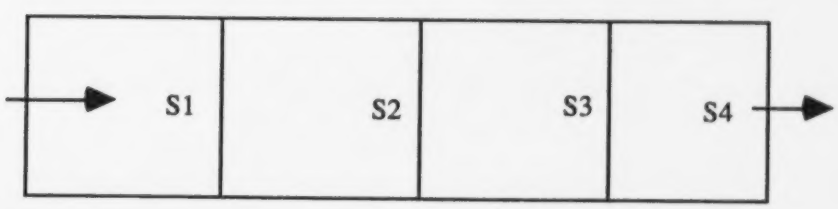


Table C.2. Hydraulic dye test on Bioreactor #1 using stock dye solution diluted 2:1 with deionized water and rotation rate of 2 rpm.

	Absorbence at 625 nm			
Elapsed time minutes	S1	S2	S3	S4
0	0.000	0.000	0.000	0.000
3	0.003	0.002	0.000	0.000
6	0.012	0.006	0.000	0.000
9	0.040	0.008	0.000	0.000
12	0.065	0.005	0.000	0.000
15	0.074	0.004	0.000	0.000
18	0.114	0.005	0.000	0.000
21	0.144	0.006	0.000	0.000
24	0.170	0.009	0.000	0.000
27	0.193	0.011	0.000	0.000
30	0.242	0.015	0.000	0.000
33	0.249	0.020	0.000	0.000
36	0.308	0.022	0.004	0.000
39	0.375	0.028	0.002	0.000
42	0.399	0.034	0.002	0.000
45	0.392	0.040	0.002	0.000
48	0.441	0.050	0.002	0.000
51	0.484	0.060	0.003	0.000
54	0.530	0.073	0.004	0.000
57	0.569	0.080	0.004	0.000
60	0.623	0.088	0.006	0.000
63	0.696	0.099	0.006	0.000
66	0.670	0.102	0.007	0.000
69	0.705	0.113	0.008	0.000
72	0.789	0.131	0.010	0.000
75	0.758	0.130	0.013	0.005
78	0.796	0.150	0.014	0.007
81	0.888	0.163	0.018	0.005
84	0.956	0.174	0.018	0.005
87	0.983	0.180	0.023	0.004
90	0.972	0.209	0.027	0.003
93	1.051	0.212	0.031	0.002
96	1.029	0.234	0.034	0.005
99	1.108	0.245	0.033	0.003
102	1.044	0.261	0.041	0.006
105	1.123	0.286	0.043	0.003
108	1.160	0.313	0.044	0.004
111	1.173	0.323	0.054	0.004
114	1.238	0.336	0.059	0.006

Table C.2 (continued). Hydraulic dye test on Bioreactor #1 (continued) using stock dye solution diluted 2:1 with deionized water and rotation rate of 2 rpm.

	Absorbence at 625 nm			
Elapsed time minutes	S1	S2	S3	S4
117	1.205	0.363	0.063	0.006
120	1.286	0.370	0.074	0.008
123	1.282	0.397	0.072	0.008
126	1.334	0.418	0.078	0.011
129	1.275	0.429	0.093	0.009
132	1.336	0.462	0.108	0.011
135	1.392	0.464	0.112	0.012
138	1.482	0.476	0.109	0.015
141	1.435	0.512	0.132	0.016
143		0.526	0.141	0.018
146		0.536	0.147	0.021
149		0.549	0.154	0.023
152		0.573	0.158	0.025
155		0.597	0.161	0.042
158		0.625	0.168	0.038
160		0.651	0.186	0.042

Figure C.2. Hydraulic dye test on Bioreactor #1 using stock dye solution and rotation rate of 1 rpm.

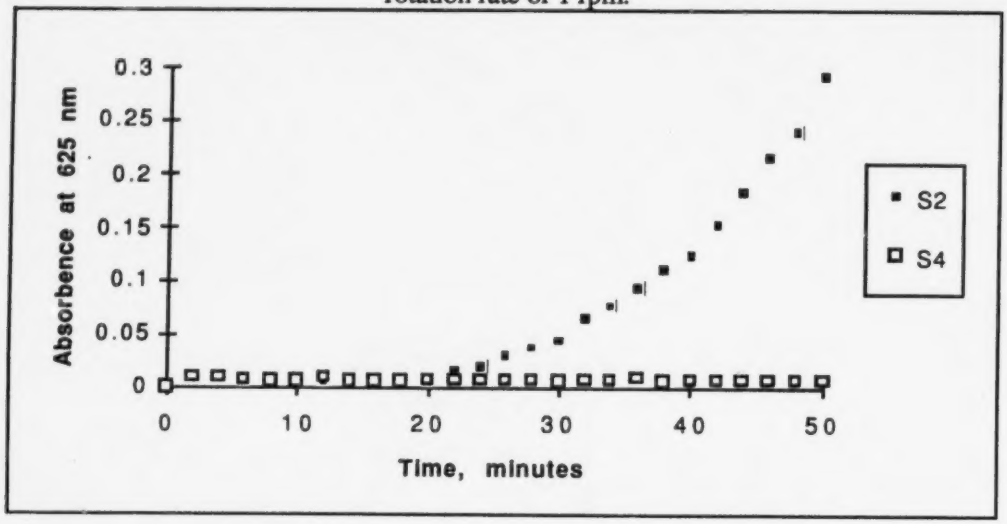
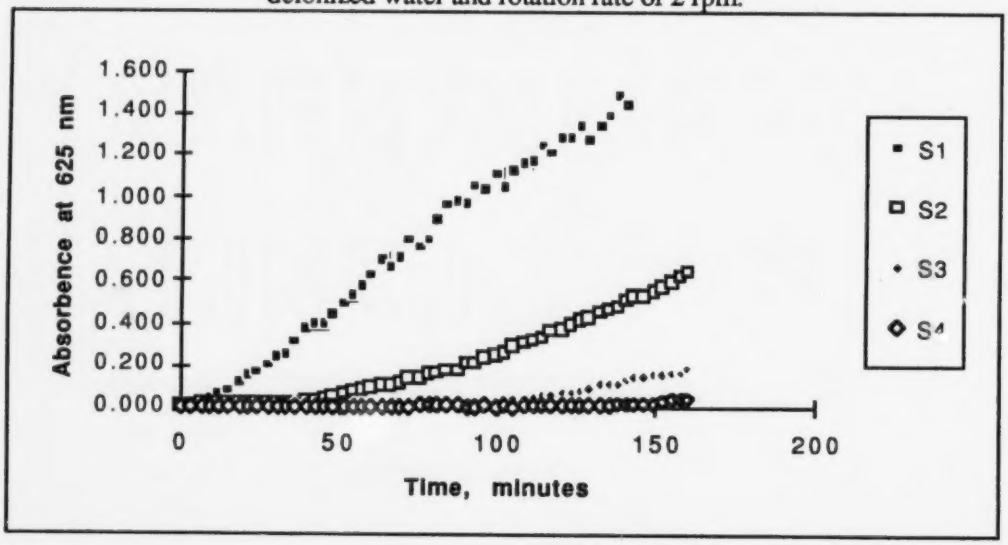


Figure C.3. Hydraulic dye test on Bioreactor #1 using stock dye solution diluted 2:1 with deionized water and rotation rate of 2 rpm.



APPENDIX D

Batch test results using suspended and attached growth systems

Glucose concentrations remaining after fungal treatment

Varying length of culture time test results

Table D.1. Batch colour test #1 colour results (CU). Suspended growth test with and without glucose and nutrients in raw and ozonated ASB effluent

Time, hours	Day	Raw 3642 N-	Raw 3642 N+	O3 3642 N-	O3 3642 N+	Raw 4251 N-	Raw 4251 N+	O3 4521 N-	O3 4521 N+
0	0	1100	1110	480	460	1100	1110	480	460
18	1	1080	1080	490	460	1060	1090	500	470
42	2	1100	990	480	430	1080	1020	480	450
66	3	1110	960	450	430	1050	990	430	330
90	4	1120	910	460	430	1060	800	440	290
114	5	1090	870	470	400	1040	650	470	290
138	6	1100	870	460	350	1070	530	460	250
162	7	1130	750	480	360	1070	470	480	250
186	8	1130	580	470	320	1080	430	480	230

Table D.1 (continued). Batch colour test #1 colour results (CU). Suspended growth test with and without glucose and nutrients in raw and ozonated ASB effluent

Time, hours	Day	Raw Control N-	Raw Control N+	O3 Control N-	O3 Control N+	Raw 24725 N-	Raw 24725 N+	O3 24725 N-	O3 24725 N+
0	0	1100	1110	480	460	1100	1110	480	460
18	1	1110	1100	490	470	1090	1070	490	470
42	2	1110	1110	490	470	1100	1020	500	440
66	3	1150	1140	500	510	1120	980	530	440
90	4	1150	1140	520	500	1120	950	530	390
114	5	1140	1150	520	510	1130	840	520	360
138	6	1150	1150	540	530	1130	670	540	330
162	7	1180	1180	550	560	1160	570	570	330
186	8	1170	1170	580	550	1160	510	550	300

Control samples have no fungus added

N+ samples contained 10 g/L glucose and same concnetration of vitamins and minerals as in growth solution

N- samples contain no glucose, vitamins or minerals

24725 referes to strain ATCC 24725

3642 refers to strain UAMH 3642

4521 refers to strain UAMH 4521

Raw is ASB effluent with no ozonation

O3 is ozonated (80 mg/L) ASB effluent

Table D.2. Batch colour test #2 colour results (CU). Attached growth test #1 with and without glucose and nutrients in raw and ozonated ASB effluent

Time, hours	Day	Raw 3642 N-	Raw 3642 N+	O3 3642 N-	O3 3642 N+	Raw 4251 N-	Raw 4251 N+	O3 4521 N-	O3 4521 N+
0	0	1140	1130	520	510	1140	1130	520	510
20	1	980	950	470	440	1020	1040	580	490
44	2	970	920	480	420	1040	1050	540	470
68	3	940	820	380	330	1040	1040	570	480
92	4	950	790	370	310	1030	1030	560	470
116	5	960	770	370	300	1040	1050	570	480
140	6	940	750	370	280	1030	1060	560	480
164	7	970	710	390	290	1060	1070	590	500
188	8	960	620	380	260	1050	1050	570	490

Table D.2 (continued). Batch colour test #2 colour results (CU). Attached growth test #1 with and without glucose and nutrients in raw and ozonated ASB effluent

Time, hours	Day	Raw Control N-	Raw Control N+	O3 Control N-	O3 Control N+	Raw 24725 N-	Raw 24725 N+	O3 24725 N-	O3 24725 N+
0	0	1140	1130	520	510	1140	1130	520	510
20	1	1120	1070	530	500	1060		480	440
44	2	1070	1070	520	500	1030	890	460	390
68	3	1120	1090	560	510	1040	770	450	310
92	4	1110	1080	560	520	1060	620	480	320
116	5	1130	1100	580	530	1060	520	480	280
140	6	1140	1090	590	530	1070	460	480	270
164	7	1160	1090	590	560	1070	410	480	240
188	8	1150	1060	600	560	1080	380	490	220

Abbreviations are the same as for Table D.1.

Table D.3. Batch colour test #3 colour results (CU). Attached growth test #2 with and without glucose and nutrients in raw and ozonated ASB effluent

Time, hours	Day	Raw 3642 N-	Raw 3642 N+	O3 3642 N-	O3 3642 N+	Raw 4251 N-	Raw 4251 N+	O3 4521 N-	O3 4521 N+
0	0	1150	1140	520	510	1150	1140	520	510
21	1	1010	970	470	430	1010	960	460	460
45	2	1030	960	360	420	1030	930	470	430
69	3	990	800	320	340	1000	830	460	420
93	4	980	760	310	290	1000	760	480	390
117	5	990	660		310	1010	610	460	310
141	6	960	580	320	300	1010	530	460	280
165	7	980	520	320	290	1010	470	480	250
189	8	1000	490	340	290	1020	430	480	250

Table D.3 (continued). Batch colour test #3 colour results (CU). Attached growth test #2 with and without glucose and nutrients in raw and ozonated ASB effluent

Time, hours	Day	Raw Control N-	Raw Control N+	O3 Control N-	O3 Control N+	Raw 24725 N-	Raw 24725 N+	O3 24725 N-	O3 24725 N+
0	0	1150	1140	520	510	1150	1140	520	510
21	1	1090	1090	510	490	1010	970	480	450
45	2	1100	1090	540	500	1010	900	500	440
69	3	1110	1080	540	500	1000	800	490	380
93	4	1120	1100	550	510	1000	690	500	350
117	5	1120	1100	560	520	1000	580	510	310
141	6	1160	1110	580	530	1020	510	520	290
165	7	1160	1110	590	520	1030	440	520	270
189	8	1150	1100	590	510	980	400	520	250

Abbreviations are the same as for Table D.1.

Table D.4. Batch colour test #3 colour results (CU). Attached growth test #2 with and without glucose and nutrients in raw and ozonated ASB effluent. Samples were run without Tween 80 addition to determine effect on decolorization.

Time, hours	Day	Raw 4521 N-	Raw 4521 N+	O3 4521 N-	O3 4521 N+
0	0	1150	1140	520	510
21	1	1040	1020	480	450
45	2	1000	950	470	390
69	3	990	870	470	390
93	4	1000	830	460	320
117	5	1000	660	450	230
141	6	1010	530	450	220
165	7	1000	470	470	210
189	8	1020	420	470	190

Table D.5. Batch colour test #4 colour results (CU). Attached growth test #3 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent. Samples were done in duplicate.

Time, hours	Day	Raw Control#1	Raw Control#2	Ozonated Control #1	Ozonated Control #2	Raw 24725 #1	Raw 24725 #2	Ozonated 24725#1	Ozonated 24725 #2
0	0	1140	1140	480	480	1140	1140	480	480
17	1	1090	1110	490	480	1040	1030	480	450
41	2	1080	1100	490	500	930	970	480	420
65	3	1070	1130	520	510	840	920	480	410
89	4	1070	1130	550	530	700	870	500	410
113	5	1050	1170	550	530	580	790	500	410
137	6	1070	1140	550	530	480	690	500	410
161	7	1090	1200	550	540	460	610	510	400

Table D.5 (continued) colour results (CU). Batch colour test #4. Attached growth test #3 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent. Samples were done in duplicate.

Time, hours	Day	Raw 3642 #1	Raw 3642 #2	Ozonated 3642 #1	Ozonated 3642#2	Raw 4521#1	Raw 4521 #2	Ozonated 4521 #1	Ozonated 4521 #2
0	0	1140	1140	480	480	1140	1140	480	480
17	1	960	990	440	440	970	980	460	450
41	2	830	880	390	420	850	980	410	400
65	3	740	730	310	340	760	970	370	360
89	4	690	620	270	320	580	950	330	330
113	5	590	520	240	280	480	900	290	300
137	6	510	470	240	260	420	860	270	270
161	7	470	470	250	260	390	840	240	260

Abbreviations are the same as for Table D.1.

Table D.6. Batch colour test #5 colour results (CU). Attached growth test #4 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in duplicate.

Time, hours	Day	Raw Control#1	Raw Control#2	Ozonated Control #1	Ozonated Control #2	Raw 24725 #1	Raw 24725 #2	Ozonated 24725#1	Ozonated 24725 #2
0	0	1100	1100	460	460	1100	1100	460	460
17	1	1030	1020	450	450	970	990	440	440
41	2	1040	1030	460	460	980	990	460	460
65	3	1010	1110	470	470	970	970	460	460
89	4	1010	1150	480	470	950	950	480	460
113	5	1050	1100	510	500	980	1000	490	480
137	6	1050	1120	450	520	990	1010	510	510
161	7	1080	1220	460	520	1020	1040	520	500

Table D.6 (continued). Batch colour test #5 colour results (CU). Attached growth test #4 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in duplicate.

Time, hours	Day	Raw 3642 #1	Raw 3642 #2	Ozonated 3642 #1	Ozonated 3642#2	Raw 4521#1	Raw 4521 #2	Ozonated 4521 #1	Ozonated 4521 #2
0	0	1100	1100	460	460	1100	1100	460	460
17	1	930	920	420	410	980	980	440	430
41	2	940	910	430	420	980	970	450	450
65	3	930	880	440	420	950	930	450	440
89	4	920	900	440	410	910	900	450	450
113	5	910	910	440	430	930	920	470	470
137	6	880	840	450	450	890	910	490	480
161	7	810	780	460	450	810	890	500	480

Abbreviations are the same as for Table D.1.

Table D.7. Batch colour test #6 colour results (CU). Attached growth test #5 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in duplicate.

Time, hours	Day	Raw Control#1	Raw Control#2	Ozonated Control #1	Ozonated Control #2	Raw 24725 #1	Raw 24725 #2	Ozonated 24725#1	Ozonated 24725 #2
0	0	1150	1150	460	460	1150	1150	460	460
20	1	1090	1080	470	490	1000	980	470	470
44	2	1100	1130	520	510	990	930	480	470
68	3	1100	1080	530	520	960	770	450	440
92	4	1050	1050	540	550	950	620	420	410
116	5	1100	1100	590	560	900	500	350	350
140	6	1110	1120	580	570	880	460	330	310
164	7	1120	1130	600	590	860	420	310	290
188	8	1130	1140	620	610	810	400	300	290

Table D.7 (continued). Batch colour test #6 colour results (CU). Attached growth test #5 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in duplicate.

Time, hours	Day	Raw 3642 #1	Raw 3642 #2	Ozonated 3642 #1	Ozonated 3642 #2	Raw 4521 #1	Raw 4521 #2	Ozonated 4521 #1	Ozonated 4521 #2
0	0	1150	1150	460	460	1150	1150	460	460
20	1	1000	1000	460	440	980	1010	430	440
44	2	980	990	450	350	910	1010	400	400
68	3	950	970	430	280	870	950	360	340
92	4	930	960	400	270	730	900	330	310
116	5	880	940	390	260	580	780	340	270
140	6	850	860	410	240	510	680	310	240
164	7	820	760	410	220	470	610	280	220
188	8	840	720	420	210	440	580	270	210

Abbreviations are the same as for Table D.1.

Table D.8. Batch colour test #7 colour results (CU). Attached growth test #6 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in triplicate.

Time, hours	Day	Raw Control	Ozonated Control	Raw 24725 #1	Raw 24725 #2	Raw 24725 #3	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3	Raw 3642 #1	Raw 3642 #2
0	0	1170	480	1170	1170	1170	480	480	480	1170	1170
20	1	1110	500	1030	1040	1030	490	440	490	1030	1030
44	2	1110	520	960	950	960	480	390	490	1020	1010
68	3	1110	540	870	860	910	440	340	500	1010	980
92	4	1120	560	720	770	850	400	280	500	1000	940
116	5	1120	580	630	660	760	340	250	520	950	900
140	6	1130	590	530	560	620	300	230	530	690	660
164	7	1160	620	470	500	560	280	220	530	580	570
188	8	1130	630	420	430	500	250	210	530	540	530

Table D.8 (continued). Batch colour test #7 colour results (CU). Attached growth test #6 with glucose in raw and ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in triplicate.

Time, hours	Day	Raw 3642 #3	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3	Raw 4521#1	Raw 4521 #2	Raw 4521 #3	Ozonated 4521 #1	Ozonated 4521 #2	Ozonated 4521 #3
0	0	1170	480	480	480	1170	1170	1170	480	480	480
20	1	1040	480	470	470	1040	1040	1060	460	480	500
44	2	1020	510	490	490	1040	1030	1040	450	500	510
68	3	1000	500	490	500	980	1000	1020	420	500	530
92	4	930	470	510	500	970	980	1000	420	490	530
116	5	830	410	520	590	970	980	1000	430	500	540
140	6	670	340	520	410	940	990	1020	440	500	560
164	7	540	290	410	360	940	1000	1010	460	510	570
188	8	470	280	320	310	940	960	990	460	490	560

Abbreviations are the same as for Table D.1.

Table D.9. Batch colour test #8 colour results (CU). Attached growth test #7 with 2, 4, or 6 g/L glucose in ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in triplicate for ATCC 24725 and UAMH 3642.

		0 g/L glucose		2 g/L glucose			4 g/L glucose			6 g/L glucose		
Time, hours	Day	Ozonated Control	Ozonated Control	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3
0	0	420	420	420	420	420	420	420	420	420	420	420
20	1	520	510	460	470	470	480	470	470	460	460	460
44	2	540	540	490	470	470	490	480	480	470	490	470
68	3	550	560	490	470	480	510	490	480	460	470	460
92	4	580	560	510	480	480	500	500	480	470	480	460
116	5	580	570	490	470	490	510	490	480	480	480	460
140	6	600	580	490	470	460	560	480	480	440	490	460
164	7	610	600	500	460	430	500	440	470	400	500	440
188	8	600	610	480	410	410	520	400	460	360	500	420

Table D.9 (continued). Batch colour test #8 colour results (CU). Attached growth test #7 with 2, 4, or 6 g/L glucose in ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in triplicate for ATCC 24725 and UAMH 3642.

		2 g/L glucose			4 g/L glucose			6 g/L glucose		
Time, hours	Day	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3
0	0	420	420	420	420	420	420	420	420	420
20	1	420	440	440	430	470	440	420	430	410
44	2	440	450	470	420	440	460	450	450	430
68	3	480	440	480	410	420	460	490	460	430
92	4	470	450	500	410	400	430	490	470	450
116	5	490	440	540	430	380	420	490	470	450
140	6	500	450	500	420	340	360	490	470	450
164	7	510	470	510	390	310	330	500	480	460
188	8	510	490	530	370	280	290	460	500	530

Table D.10. Batch colour test #9 colour results (CU). Attached growth test #8 with 2, 4, or 6 g/L glucose in ozonated ASB effluent. No vitamins or minerals were added to the pulp mill effluent and the effluent was not heat treated before being used. Samples were done in triplicate for UAMH 4521.

		0 g/L glucose		2 g/L glucose			4 g/L glucose			6 g/L glucose		
Time, hours	Day	Ozonated Control	Ozonated Control	Ozonated 4521 #1	Ozonated 4521 #2	Ozonated 4521 #3	Ozonated 4521 #1	Ozonated 4521 #2	Ozonated 4521 #3	Ozonated 4521 #1	Ozonated 4521 #2	Ozonated 4521 #3
0	0	430	430	430	430	430	430	430	430	430	430	430
20	1	450	460	440	430	430	430	430	430	470	450	440
44	2	500	490	470	460	460	420	440	400	460	450	440
68	3	510	500	480	450	460	390	450	370	440	440	450
92	4	510	500	470	450	460	350	450	330	370	430	460
116	5	530	510	490	440	450	300	400	280	320	410	450
140	6	540	540	490	450	460	300	350	260	300	410	430
164	7	540	550	480	440	430	260	320	230	270	390	370
188	8	540	590	470	420	380	240	300	210	260	340	320

Table D.11. Glucose concentrations (g/L) from Attached growth test #7 with 2, 4, and 6 g/L glucose in ozonated pulp mill effluent

		0 g/L glucose		2 g/L glucose			4 g/L glucose			6 g/L glucose		
Time, hours	Day	Ozonated Control	Ozonated Control	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3	Ozonated 24725 #1	Ozonated 24725 #2	Ozonated 24725 #3
0	0	0.00	0.00	2.02	2.02	2.02	4.04	4.04	4.04	6.07	6.07	6.07
44	2	0.00	0.00	1.95	1.96	1.95	3.82	3.80	3.77	5.58	5.66	5.66
92	4	0.00	0.00	1.64	1.68	1.77	3.71	3.69	3.77	5.58	5.64	5.66
140	6	0.00	0.00	1.42	1.46	1.55	3.62	3.44	3.55	5.31	5.49	5.39
188	8	0.00	0.00	1.24	1.19	1.28	3.46	3.35	3.39	5.12	5.31	5.24

Table D.11 (continued). Glucose concentrations (g/L) from Attached growth test #7 with 2, 4, and 6 g/L glucose in ozonated pulp mill effluent

		2 g/L glucose			4 g/L glucose			6 g/L glucose		
Time, hours	Day	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3	Ozonated 3642 #1	Ozonated 3642 #2	Ozonated 3642 #3
0	0	2.02	2.02	2.02	4.04	4.04	4.04	6.07	6.07	6.07
44	2	1.33	1.33	1.37	3.33	3.35	3.31	5.06	5.33	5.19
92	4	0.99	0.95	0.92	2.86	2.88	2.90	4.92	4.81	4.76
140	6	0.74	0.65	0.59	2.59	2.56	2.50	4.50	4.41	4.22
188	8	0.40	0.40	0.31	2.11	2.25	2.07	4.20	4.05	3.84

Table D.12. Colour removal at varying length of culture time analysis table. Samples were transferred to ozonated (80 mg/L) pulp mill effluent after culture times of 2 to 9 days. Results are in colour units (CU)

	2 day growth		3 day growth		4 day growth		5 day growth		6 day growth		7 day growth		8 day growth		9 day growth	
Day	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B	6A	6B	7A	7B	8A	8B
0	290	290	290	290	290	290	290	290	290	290	290	290	290	290	290	290
2	310	310	300	280	290	240	290	240	250	230	240	230	270	260	220	230
4	290	250	280	200	250	190	230	200	240	140	230	170	140	260	180	220
6	190	150	230	110	160	100	150	100	160	90	200	100	90	240	100	140
8	120	90	120	80	110	70	130	80	120	80	200	90	70	240	100	110
% removal	58.6%	69.0%	58.6%	72.4%	62.1%	75.9%	55.2%	72.4%	58.6%	72.4%	31.0%	69.0%	75.9%	17.2%	65.5%	62.1%
CU/day	21	25	21	26	23	28	20	26	21	26	11	25	28	6	24	23
Avg % removal	63.8%		65.5%		69.0%		63.8%		65.5%		50.0%		46.6%		63.8%	
Avg CU/day	23		24		25		23		24		18		17		23	

APPENDIX E

Colour change after ozonation tests including colour, BOD, pH, and HPC results from the first test at variable temperatures and from the second test using a factorial design to evaluate temperature and pH effects

Table E.1. Colour change (CU) after ozonation test #1

Date	Time, hrs	4 degrees C			22 degrees C			40 degrees C		
		#1	#2	#3	#1	#2	#3	#1	#2	#3
6-Nov	0	320	320	320	320	320	320	320	320	320
7-Nov	24	290	290	290	310	320	320	360	370	360
8-Nov	48	300	300	300	330	340	330	380	390	380
9-Nov	72	290	290	290	330	340	330	380	380	370
10-Nov	96	300	300	290	330	340	330	370	370	360
11-Nov	120	300	300	290	330	340	330	370	370	360
12-Nov.	144	300	300	300	340	350	330	370	370	370
13-Nov	168	280	300	290	330	330	330	360	360	360
14-Nov	192	290	290	290	340	340	340	370	370	370
15-Nov	216	290	290	280	340	340	330	370	360	360
16-Nov	240	290	290	280	330	330	330	360	350	350

Table E.2. pH change results in ozonated colour change after ozonation test #1

Date	Time, hrs	4 degrees C			22 degrees C			40 degrees C		
		#1	#2	#3	#1	#2	#3	#1	#2	#3
6-Nov	0	7.79	7.79	7.79	7.79	7.79	7.79	7.79	7.79	7.79
7-Nov	24	8.27	8.18	8.20	8.32	8.31	8.51	8.55	8.47	8.47
8-Nov	48	8.37	8.42	8.45	8.54	8.41	8.70	8.89	8.83	8.85
9-Nov	72	8.67	8.64	8.70	8.73	8.65	8.75	9.10	8.98	9.02
10-Nov	96	8.71	8.72	8.82	8.84	8.74	8.79	9.12	9.01	9.03
11-Nov	120	8.69	8.66	8.68	8.84	8.82	8.88	9.13	9.08	9.08
12-Nov	144	8.70	8.66	8.71	8.81	8.77	8.83	9.20	9.05	9.13
13-Nov	168	8.79	8.84	8.84	8.77	8.81	8.92	9.23	9.18	9.22
14-Nov	192	8.84	8.92	8.92	8.90	8.97	8.99	9.32	9.27	9.29
15-Nov	216	8.98	9.00	9.01	8.93	9.07	9.09	9.39	9.35	9.38
16-Nov	240	9.04	9.05	9.08	8.87	9.04	9.15	9.40	9.36	9.35

Table E.3. BOD (mg/L) change results in ozonated colour change test #1

Date	Time, hrs	4 degrees C			22 degrees C			40 degrees C		
		#1	#2	#3	#1	#2	#3	#1	#2	#3
6-Nov	0	19.56	19.56	19.56	19.56	19.56	19.56	19.56	19.56	19.56
10-Nov	96	22.06	22.11	23.71	13.68	12.52	14.08	13.22	14.28	14.01
14-Nov	144	22.97	23.38	24.99	9.44	8.21	8.36	4.64	4.83	6.80
12-Nov	192	23.01	22.49	21.37	5.73	5.19	5.53	4.01	3.25	3.58
13-Nov	240	23.77	23.83	24.24	4.52	3.90	3.28	3.59	1.77	2.76

Table E.4. Heterotrophic plate count (cfu/mL) change results in ozonated colour change test #1. HPC at 35°C for 48 hours

Time, hours	Raw initial	Ozonated initial	4 °C composite	22 °C composite	40 °C composite
0	1.00E+05	2.00E+04	2.00E+04	2.00E+04	2.00E+04
120			9.30E+03	8.10E+05	1.85E+06
240			1.73E+04	4.13E+06	7.70E+05

Table E.5. Heterotrophic plate count (cfu/mL) change results in ozonated colour change test #1. HPC at 20°C for 7 days

Time, hours	Raw initial	Ozonated initial	4 °C composite	22 °C composite	40 °C composite
0	2.43E+05	1.63E+05	1.63E+05	1.63E+05	1.63E+05
120			2.00E+04	1.00E+06	1.60E+06
240			2.40E+04	3.57E+06	2.43E+04

Table E.6. Colour Change (CU) results in ozonated colour change test #2

Date	Time, hrs	4 degrees C				22 degrees C			40 degrees C			
		Low pH #1	Low pH #2	High pH #1	High pH #2	Mid pH #1	Mid pH #2	Mid pH #3	Low pH #1	Low pH #2	High pH #1	High pH #2
23-Nov	0	460	460	460	460	460	460	460	460	460	460	460
24-Nov	24	430	470	450	450	460	460	460	490	490	520	520
25-Nov	48	450	450	440	440	470	460	460	500	490	510	510
26-Nov	72	390	400	420	430	470	470	470	550	500	500	500
27-Nov	96	410	410	440	450	480	470	470	560	540	510	510
29-Nov	144	420	410	440	440	480	480	480	620	600	500	490
1-Dec	192	420	410	430	430	470	470	470	620	590	470	470
3-Dec	240	390	410	430	430	470	470	470	620	590	470	460

Table E.7. pH results in ozonated colour change test #2

Date	Time, hrs	4 degrees C				22 degrees C			40 degrees C			
		Low pH #1	Low pH #2	High pH #1	High pH #2	Mid pH #1	Mid pH #2	Mid pH #3	Low pH #1	Low pH #2	High pH #1	High pH #2
23-Nov	0	4.00	4.00	7.20	7.20	5.60	5.60	5.60	4.00	4.00	7.20	7.20
24-Nov	24	3.94	3.93	8.15	8.06	7.52	7.58	7.61	3.97	4.02	8.46	8.49
25-Nov	48	4.03	3.97	8.35	8.36	7.58	7.60	7.64	4.05	4.06	8.67	8.70
26-Nov	72	3.98	3.96	8.35	8.46	7.56	7.65	7.69	4.01	3.99	8.92	8.91
27-Nov	96	4.14	4.01	8.33	8.38	7.44	7.45	7.46	4.13	4.10	8.91	8.89
29-Nov	144	4.02	3.96	8.62	8.72	8.13	8.25	8.35	4.29	4.21	9.12	9.15
1-Dec	192	4.00	3.93	8.75	8.82	8.30	8.46	8.52	4.25	3.61	9.20	9.22
3-Dec	240	3.94	3.91	8.75	8.77	8.13	8.40	8.45	4.19	4.19	9.27	9.30

Table E.8. BOD (mg/L) results in ozonated colour change test #2

Date	Time, hrs	4 degrees C				22 degrees C			40 degrees C			
		Low pH #1	Low pH #2	High pH #1	High pH #2	Mid pH #1	Mid pH #2	Mid pH #3	Low pH #1	Low pH #2	High pH #1	High pH #2
23-Nov	0	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
25-Nov	48	26.90	29.51	31.84	32.70	24.08	24.29	24.63	28.01	27.29	8.31	8.43
29-Nov	144	24.59	25.63	28.62	28.59	7.67	5.94	5.58	20.90	18.82	4.35	4.43
3-Dec	240	27.04	27.52	28.91	30.06	2.87	2.95	2.65	18.15	16.31	4.46	4.30
3-Dec *	240	27.16	26.86	25.35	25.05	1.74	1.02	1.17	16.49	15.46	0.96	0.93

* second BOD result for December 3 1995 is soluble BOD rather than total BOD as in the preceding samples

Table E.9. Heterotrophic plate count(cfu/mL) change results in ozonated colour change test #2. HPC at 35°C for 48 hours with composite samples

Date	Time, hrs	4 degrees C		22 degrees C	40 degrees C	
		Low pH	High pH	Mid pH	Low pH	High pH
23-Nov	0	3.0E+04	3.0E+04	3.0E+04	3.0E+04	3.0E+04
29-Nov	144	1.2E+03	2.6E+03	5.1E+04	1.0E+03	1.9E+06
3-Dec	240	4.6E+01	2.3E+04	7.5E+05	1.6E+03	4.0E+05

Table E.10. Heterotrophic plate count(cfu/mL) change results in ozonated colour change test #2. HPC at 20°C for 7 days with composite samples

Date	Time, hrs	4 degrees C		22 degrees C	40 degrees C	
		Low pH	High pH	Mid pH	Low pH	High pH
23-Nov	0	5.1E+05	5.1E+05	5.1E+05	5.1E+05	5.1E+05
29-Nov	144	4.9E+03	6.0E+04	1.3E+06	7.0E+02	1.5E+06
3-Dec	240	2.7E+03	1.2E+05	3.8E+06	1.9E+03	5.9E+05

APPENDIX F

Batch tests results with steel plates present

Batch test results with added metals

Table F.1. Steel toxicity test #1. Colour changes after incubation with fungus and steel plates. Results in colour units (CU)

			No steel		Steel in growth and effluent		Steel in growth, no steel in effluent		No steel in growth, steel in effluent	
Time, days	Time, hours	Control	1A	1B	2A	2B	3A	3B	4A	4B
0	0	460	460	460	460	460	460	460	460	460
2	48	530	310	240	300	270	300	270	270	270
4	96	510	250	180	210	210	230	200	160	200
6	144	500	190	140	190	150	180	160	160	190
Average	144	500	165		170		170		175	
Average 6 day % removal			64.1%		63.0%		63.0%		62.0%	

Table F.2. Steel Toxicity test #2. Colour changes after incubation with fungus and steel plates or welding rods. Results in colour units (CU)

		No steel		Welded steel in growth and effluent		Welding rod pieces in growth and effluent	
Time, days	Time, hours	1A	1B	2A	2B	3A	3B
0	0	460	460	460	460	460	460
2	48	310	240	300	270	300	270
4	96	250	180	210	210	230	200
6	144	190	140	190	150	180	160
8	168	120	110	240	310	130	110
Average	0	400		400		400	
	48	245		450		220	
	96	185		470		195	
	144	135		355		150	
Average 6 day % removal		66.3%		11.3%		62.5%	

Table F.3. Steel Toxicity test #3. Colour changes after incubation with fungus and steel plates. Results in colour units (CU)

		No steel			Welded steel in growth and effluent		
Time, days	Time, hours	1A	1B	1C	2A	2B	2C
0	0	440	440	440	440	440	440
2	48	310	320	300	330	340	320
4	96	190	210	190	210	200	190
6	144	190	180	180	210	190	200
Average	144	183			200		
Average 6 day % removal		58.3%			54.5%		

Table F.4. Steel Toxicity test #4. Colour changes after incubation with fungus and steel plates. Results in colour units (CU)

		No steel			Welded steel in growth and effluent		
Time, days	Time, hours	1A	1B	1C	2A	2B	2C
0	0	420	420	420	420	420	420
2	48	250	220	260	220	210	220
4	96	190	170	210	210	160	170
6	144	150	140	150	150	140	130
Average	144	147			140		
Average 6 day % removal		65.1%			66.7%		

Table F.5. Metal concentration test #1. Colour changes after incubation with fungus and metals. Results in colour units (CU)

	Control		10.5 mg/L Iron		0.5 mg/L Chromium		2 mg/L Nickel		0.06 mg/L Copper		All metals	
Time, hours	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2
0	440	440	440	440	440	440	440	440	440	440	440	440
48	410	370	670	660	360	360	370	430	430	370	610	650
96	420	210	420	430	240	220	210	230	230	310	520	560
144	200	150	430	380	170	160	140	150	180	200	480	510
Average % reduction	60%		8%		63%		67%		57%		-13%	

Table F.6. Metal concentration test #2. Colour changes after incubation with fungus and metals. Results in colour units (CU)

	Control		10.5 mg/L Iron		0.5 mg/L Chromium		2 mg/L Nickel		0.06 mg/L Copper		All metals	
Time, hours	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2	Sample #1	Sample #2
0	440	440	440	440	440	440	440	440	440	440	440	440
48	280	290	610	630	290	280	310	330	360	380	610	620
96	170	170	650	630	180	160	210	190	190	190	620	640
144	130	130	500	710	160	120	180	150	190	230	600	640
Average % reduction	70%		-38%		68%		63%		52%		-41%	

Table F.7. Metal concentration test #2. Control colour changes in colour units (CU)

	Control colour increase - no fungus		
Time, hours	No metals	All metals	Iron
0	440	440	440
48	500	620	700
96	550	670	720
144	590	720	770
Average % reduction	-34%	-64%	-75%

APPENDIX G

Batch tests for evaluation of improved media in both raw and ozonated pulp mill effluent

Table G.1. Improved media evaluation with ozonated ASB pulp mill effluent. Sample identification key is provided at the end

		Time, hours				
		0	24	72	120	168
A	Sample 1	400	350	320	340	270
	Sample 2	400	380	360	360	290
B	Sample 1	400	400	200	190	180
	Sample 2	400	510	430	460	380
C	Sample 1	400	440	500	500	470
	Sample 2	400	440	530	530	550
D	Sample 1	400	450	530	570	410
	Sample 2	400	450	530	590	580
E	Sample 1	400	320	320	280	250
	Sample 2	400	320	240	230	190
F	Sample 1	400	370	200	180	160
	Sample 2	400	230	135	120	110
G	Sample 1	400	400	440	370	330
	Sample 2	400	430	430	330	300
H	Sample 1	400	490	400	410	350
	Sample 2	400	410	480	600	650
I	Sample 1	400	420	370	420	340
	Sample 2	400	260	240	250	210
	Sample 3	400	370	360	330	320

Table G.2. Improved media evaluation with raw ASB pulp mill effluent. Sample identification key is provided at the end

		Time, hours			
		0	72	120	168
A	Sample 1	1150	330	310	290
	Sample 2	1150	340	270	270
B	Sample 1	1150	270	230	210
	Sample 2	1150	270	240	200
C	Sample 1	1150	660	580	620
	Sample 2	1150	660	550	570
D	Sample 1	1150	680	570	580
	Sample 2	1150	780	590	560
E	Sample 1	1150	340	310	260
	Sample 2	1150	320	320	280
F	Sample 1	1150	330	290	230
	Sample 2	1150	520	400	250
G	Sample 1	1150	580	520	470
	Sample 2	1150	560	500	490
H	Sample 1	1150	620	410	410
	Sample 2	1150	690	440	450
I	Sample 1	1150	530	500	490
	Sample 2	1150	680	490	450
	Sample 3	1150	500	470	460

Identification key for samples in raw and ozonated improved media factorial test

	Tween 80	Buffer	Manganese
A	-	-	-
B	+	-	-
C	-	+	-
D	+	+	-
E	-	-	+
F	+	-	+
G	-	+	+
H	+	+	+
I	0	0	0

Table G.3. Improved media evaluation and comparison with glucose added samples. 10 ± 0.5 g wet weight of fungus was added to 150 mL wastewater in each sample

	Improved Media				Sponges + glucose + buffer				Glucose + buffer			
Time, days	Raw #1	Raw #2	O3 #1	O3 #2	Raw #1	Raw #2	O3 #1	O3 #2	Raw #1	Raw #2	O3 #1	O3 #2
0	1220	1220	460	460	1220	1220	460	460	1220	1220	460	460
1	1090	890	460	340	910	930	430	390	1030	1080	460	440
3	950	590	490	250	790	800	280	320	630	920	320	300
5	770	470	450	210	760	750	270	300	480	560	260	260
7	850	440	430	190	810	790	210	260	380	430	200	210
% removal	30.3%	63.9%	6.5%	58.7%	33.6%	35.2%	54.3%	43.5%	68.9%	64.8%	56.5%	54.3%
average removal	47.1%		32.6%		34.4%		48.9%		66.8%		55.4%	
CU removed	370	780	30	270	410	430	250	200	840	790	260	250
Avg CU removed	575		150		420		225		815		255	